IMMUNOLOGICAL ASPECTS OF HERPESVIRUSES IN HUMAN NEOPLASIA

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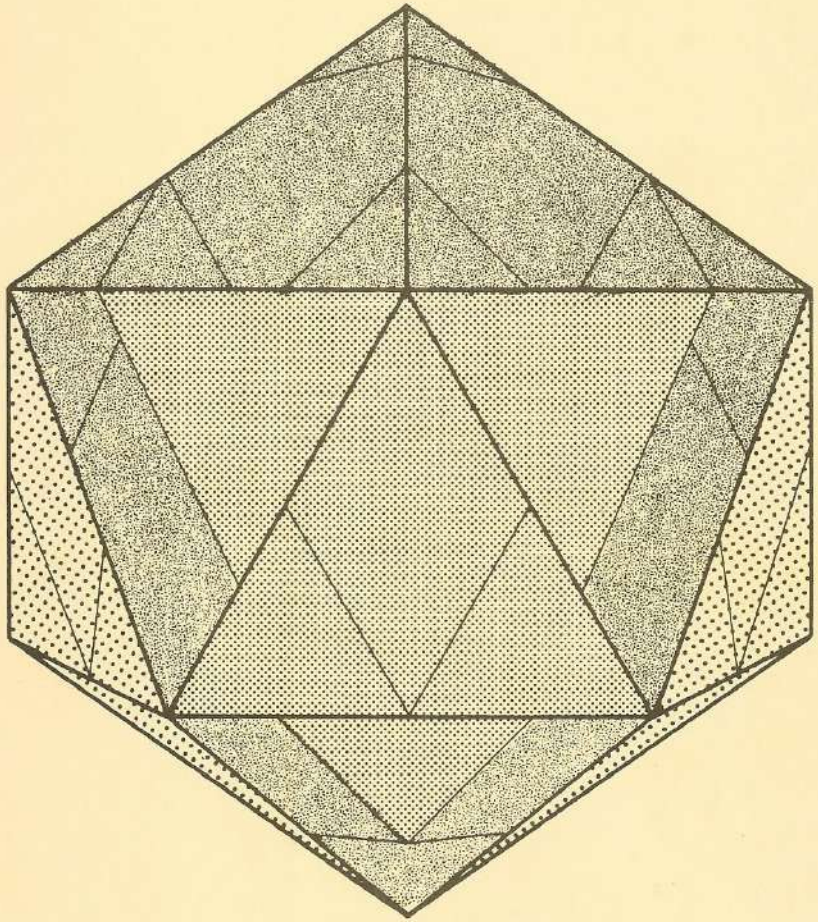

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Academisch proefschrift

ter verkrijging van de graad van doctor in de Geneeskunde
aan de Universiteit van Amsterdam, op gezag van de
Rector Magnificus Dr. G. den Boef, hoogleraar in de
Faculteit der Wiskunde en Natuurwetenschappen,
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door

Franciscus Johannes Marie Hilgers
geboren te Beek (L)

1976
MONDEEL-OFFSETDRUKKERIJ
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Omslagontwerp: Chris Bor.

Het is beter één enkel lampje aan te steken dan de duisternis te vervloeken.

Confucius (551-479 voor Chr.)

Promotor: Prof. Dr. F. Dekking
Co-promotor: Prof. Dr. F.J. Cleton
Co-referent: Dr. T.H. The

voor mijn overleden vader
mijn moeder
agnes

CONTENTS

VOORWOORD

Hooggeleerde Dekking, hooggeachte promotor, met genoegen denk ik terug aan de tijd dat U mij inwijdde in de beginselen van de weefselkweek en de herpesvirologie. Hoewel het onderzoek aanvankelijk een andere richting uitging, hebt U toch altijd stimulerende waardering kunnen opbrengen voor de veranderde opzet van dit proefschrift. Zonder de uitnemende faciliteiten van Uw laboratorium zouden belangrijke delen van dit werk niet tot stand gekomen kunnen zijn.

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Zeergeleerde Hilgers, beste Jo, slechts weinigen zullen beseffen hoe belangrijk jouw bijdrage aan dit proefschrift is. Niet alleen als co-auteur van 2 artikelen, maar als motor achter al mijn wetenschappelijke activiteiten. Wat begon als een klein en vrijblijvend uitstapje vanuit het relatief beschermde klinische leven naar de "woeste" wetenschappelijke wereld, is voor mij uitgegroeid tot een volledig nieuw inzicht over de functie van de medicus. De combinatie van research en kliniek lijkt nu een vanzelfsprekende en obligaat voor een zinvol functioneren als arts. Ook in de toekomst zal de samenwerking tussen de bioloog en de medicus in de familie zeker goede vruchten afwerpen.

Hooggeleerde Jongkees, in de afgelopen 2 jaar als klinisch assistent in Uw kliniek is mij duidelijk geworden dat de combinatie van kliniek en research voor U een vanzelfsprekende zaak is. Iets wat elders nog te zelden in praktijk wordt gebracht. Door Uw niet aflatende belangstelling voor de vorderingen van mijn proefschrift en door de tijd die U als studieverlof ter beschikking stelde, lukte het mij naast het klinische werk dit proefschrift te voltooien. Het is mij daarom ook een eer mijn KNO-opleiding in Uw kliniek te mogen ontvangen.

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De rol, die de ouders spelen in de loopbaan van hun kinderen, is niet

altijd even duidelijk en wordt vaak onderschat. Ik weet echter dat mijn ouders zeer bewust aangestuurd hebben op een zo optimaal mogelijke opleiding van hun kinderen en het behoeft geen betoog dat ik zonder hun hardnekkige inspanning niet zover zou zijn gekomen. Ik ben hen daar zeer dankbaar voor.

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ONCOGENIC HERPESVIRUSES

General introduction

The herpesviruses form a large group of pathogenic micro-organisms found in a wide variety of animal species. Approximately 43 viruses are known to date. Many different clinical pictures can be caused by infection with these viruses, ranging from such relative mild conditions as stomatitis to highly fatal malignancies. This latter aspect of their properties will be the main topic of this review.

The definition of herpesviruses according the International Committee on Nomenclature of Viruses, 1970, is as follows: "the viruses contain double-stranded DNA with a molecular weight of 54 to 92×10^6 Daltons. *G*(uanine) + *C*(ytosine) content varies from 57 to 74%. Virus particles range from about 100 to 150 nm in diameter. The virus contains a capsid which is icosahedral with 162 hollow capsomeres and a diameter of 100 nm and with a lipid containing membrane and therefore sensitive to lipid solvents. The DNA is about 7% of the particle weight. The buoyant density in CsCl gradients varies from 1.17 to 1.29 gr/cm^3. Development begins in the nucleus and is completed by the addition of protein membranes as the virus passes into the cytoplasm. Intranuclear inclusion bodies can be found".

The most intruiging aspect of virus-cell relationship of the herpesviruses is their ability to infect in two different ways:
1) productive infection which leads to the synthesis of structural viral components, the assembly of viral progeny and invariably to cell death,
2) non-productive infection without the synthesis of structural viral components. In this case the cell survives, maintaining the viral genome. This results in a persistent and latent presence of the virus after primary infection.

The ability to infect in a non-productive way appears to be a common characteristic of all herpesviruses. Cells infected in this way can be induced to produce virus, which will lead to cell death. In case of the oncogenic herpesviruses the events that lead to malignant transformation will only be effective in non-productively infected cells, since full expression of viral functions will result in viral progeny and in cell death. The expression of these viral properties depends on the type of cell which is infected:
1) permissive cells are cells allowing a complete productive cycle and are killed in the process,
2) non-permissive cells possess restriction mechanisms capable to suppress certain viral functions and are not killed by the virus.

Some herpesviruses have oncogenic properties. This has been proven in several animal models. In man at least one of the herpesviruses, Epstein-Barr virus (EBV), is a possible example in this respect.

1. *Lucké tumor-associated herpesvirus (LHV)*

The Lucké tumor is a renal adenocarcinoma in the common leopard frog (Rana pipiens), first described by Lucké in 1934. This is the oldest tumor known to be associated with a herpesvirus. It was observed that during hibernation in tumors of frogs Cowdry type A intranuclear inclusion bodies could be found. These inclusion bodies appeared to be invariably related with the presence of herpesvirus particles, as could be demonstrated by electron microscopy.

The tumor fulfills all the criteria for a malignant neoplasm, that is, local invasion and distant metastasis are normally occurring events. There is a strong relationship between seasonal variations in temperature and presence of herpesviruses in the tumor. At low temperature, during hibernation or 4-9°C under laboratory conditions, virus particles can be found in so called "cold" tumors in up to 30% of the tumor cells, as well as in the urine and in ascitic fluid. Tumors of frogs captured during the summer season or kept at 20-22°C under laboratory conditions (so called "warm" tumors) are always free of mature virus. However, they contain the LHV genome in the nucleus of the tumor cells, as could be demonstrated by nucleic acid hybridization. A temperature shift from "cold" to "warm", or the reversed, changes virus positive tumors into virus negative and vice versa. This phenomenon can be considered as a natural example of a temperature sensitive herpesvirus. The common characteristic for herpesviruses, that productive infection leads to cell death, is clear. Virus induction by low temperature causes cell damage and reduction of the size of the tumor.

Cellfree, crude or semipurified virus preparations of LHV, obtained from "cold" tumors, induce typical Lucké tumors in developing frog embryos, even if kept at summer temperature. Similar extracts from "warm", virus negative tumors do not induce tumors. From these infection experiments it is clear that the natural target cell for LHV is the differentiating embryonic kidney cell and that the fully differentiated kidney cell is not susceptible to virus infection. This means that the period in which the infection will ultimately lead to tumor development is a critical and very short one.

Some restrictions about a causal relationship of LHV with the renal adenocarcinoma of the frog can be made, because of the occasional presence of at least 3 other viruses in the tumor, one of which is also a herpesvirus. However, up till now no similar induction and transmission experiments, as described for LHV, could be performed with these viruses.

2. *Marek's disease herpesvirus (MDHV)*

This herpesvirus causes a lymphoproliferative disease in domestic chicken. Two forms of the disease can be distinguished: (1) a classical or chronic form, first described by Marek in 1907, characterized primarily by involvement of peripheral nerves, leading to paralysis, *e.g.* of legs and wings, and (2) a more acute form, characterized by visceral lymphoid tumors.

MDHV appears to be a ubiquitous virus among the poultry populations of the world. All known commercial lines of chicken have been shown to be more or less susceptible to infection with the virus, but the genetic constitution has a great influence on the incidence of Marek's disease after infection. MDHV can cause a self-limiting lymphoproliferative disease, a highly malignant lymphoma or no disease at all. The mortality varies from practical zero up to 70-80%.

The virus causes a productive infection in the epithelial cells of the feather follicle. This is probably the source of virus for the horizontal spread in the poultry population. All other cells and in particular lymphoid cells and cells of lymphomas are non-productively infected. However, in some cases a productive, but abortive infection may be found here also.

MDHV can be grown in cultured embryo cells of many avian species. These can be used as indicator cells, for by co-cultivation with non-productively infected cells a cytopathic effect is produced by the activated virus.

Cellfree preparations of MDHV, prepared from feather follicle epithelium, have been shown to produce Marek's disease. Lymphoblastoid cell lines with T-cell characteristics can be established at 41°C by culturing tumor cells from infected birds. By nucleic acid hybridization and immunofluorescence it is possible to demonstrate the presence of viral DNA and virus-induced antigens in these cell lines.

There is a wide range in pathogenicity of the different strains or isolates of MDHV, even in the most susceptible lines of domestic chicken. Some isolates are fully pathogenic and produce the acute and most severe form of the disease, others are less pathogenic and produce the classical form and still others are non-pathogenic. Serological classifications are in concordance with this and 3 subtypes can be distinguished: (1) pathogenic strains of MDHV and attenuated strains, (2) apathogenic strains of MDHV and (3) Herpesvirus Turkey (HT) and attenuated variants. HT is a serologically distinct, but closely related herpesvirus, isolated from turkeys. This virus is non-pathogenic to chickens and turkeys and can be used for vaccination of susceptible chickens. It appears that this vaccination does not prevent (super)infection with MDHV, but protects the infected animal for the subsequent development of either forms of Marek's disease. Besides HT, attenuated strains of pathogenic types of MDHV can be used for vaccination also.

The role of cellular and humoral immunity in the development of a malignant lymphoma is not quite clear. Thymectomy or bursectomy, or both, do not influence the incidence of Marek's disease in susceptible chickens, whereas thymectomy alone somewhat increases the incidence in genetically resistant birds. This might indicate that genetical resistance is partly due to cellular immunity.

3. *Herpesvirus Saimiri (HVS) and herpesvirus Ateles (HVA)*

Herpesvirus Saimiri was isolated in 1968 by Melendez from a spontaneous degenerating kidney cell culture of a squirrel monkey. Whereas the virus does not cause any overt disease in the natural hosts, the squirrel and spider monkeys, it produces a highly fatal malignant lymphoma or reticulum cell tumor in various other subhuman primates like marmosets and owl monkeys. New Zealand white rabbits have been shown to be susceptible to HVS malignant lymphoma induction as well.

Virus particles or antigens cannot be demonstrated in tumor cells, but by co-cultivation with indicator cells viral antigens appear and the virus can readily be recovered. The same method can be used to demonstrate the presence of viral DNA in peripheral lymphocytes, spleen cells and lymph-nodes from antibody positive animals.

Permanent lymphoblastoid cell lines with T-cell characteristics can be established from lymphomas. Various degrees of antigen expression can be found in these cell lines, but ultimately they become non-producer lines after serial passage. In contrast to herpesvirus Ateles (HVA) and EBV, to be discussed later, HVS is not capable to transform lymphocytes *in vitro*, although it causes a malignant lymphoma *in vivo*.

After infection in tissue culture of susceptible indicator cells two different antigens can be distinguished: (1) early viral antigen (EA), appearing after inhibition of the DNA synthesis by cytosin arabinoside, and (2) late viral antigen (LA) which appears when the viral cycle is completed.

Antibodies to these antigens can be demonstrated in infected animals and show a remarkable pattern. After infection of squirrel monkeys antibodies to both LA and EA develop in about 2 weeks time. Whereas antibody titers to LA tend to be stable throughout life, titers to EA decline to undetectable levels after several months. Marmosets and owl monkeys, reacting with malignancies to infections with HVS, also produce antibodies to LA and EA, but detectable levels are not found before the 4th week after infection. Furthermore, antibodies to EA generally remain present at high levels until death. This is a situation quite similar to what can be observed in EBV-related malignancies, *i.e.* Burkitt's lymphoma and nasopharyngeal carcinoma.

Sero-epidemiology of HVS has shown that this is a ubiquitous virus among squirrel monkeys. Almost all animals captured in nature possess antibodies to LA and are negative to EA. Experimental infection of sero-negative adult squirrel monkeys does not result in any clinical sign of illness. In comparison with EBV the latter observation is in contrast to the high frequency of infectious mononucleosis (IM) as a result of infection at adolescence with EBV, although the antibody pattern is quite similar in both cases.

Like in Marek's disease, where active vaccination, as described, has proven to be very effective in its prevention, experiments to vaccinate with HVS are in progress. Both attenuated virus and inactivated virus have already been used with some success in the prevention of tumor induction in susceptible monkey species by HVS.

HVA was isolated from a healthy spider monkey and it appears to behave very similar to HVS in its natural history and pathogenesis, although both viruses are immunological clearly distinct. The spider monkey is the natural host of HVA and here the infection is asymptomatic. In several other subhuman primates, like marmosets and owl monkeys, infection with this virus leads to the development of a highly fatal lymphoma. Unlike HVS, as already mentioned, HVA readily transforms T-lymphocytes *in vitro*.

Besides the 4 oncogenic herpesviruses, described above, several other herpesviruses are suspected of oncogenic capacities. Knowledge about these viruses is limited. The most important example seems to be herpesvirus Sylvilagus, probably causing a (self-limiting?) lymphoproliferative disease in wild contton-tail rabbits. A herpesvirus could be isolated from a guinea pig leukemia, but here a causal relationship is even less evident. In lower vertebrates a herpesvirus has been associated with Epithelioma Papulosum or "carp pox", a benign dermal lesion in the cultured carp (Cyprinus carpio).

4. *Epstein-Barr virus (EBV)*

In 1964 Epstein, Achong and Barr demonstrated by electron microscopy the presence of herpesvirus like particles in cultured tumor cells derived from a Burkitt's lymphoma. This malignant lymphoma was first described by the British surgeon Burkitt, who discovered the tumor in Africa, where young children are struck by this disease. There appeared to be time and space clustering in areas with hyper- and holoendemic malaria, reasons to theorize about a vectored virus to be the causal agent for the disease. Culturing these tumor cells virtually always results in continually growing lymphoblastoid cell lines. Some of these cell lines show herpesvirus particles, whereas others do not.

Antibodies can be detected by an indirect immunofluorescence technique, using virus containing lymphoblastoid cell lines (Henle, 1966). The majority of sera from adults show positive reactions. These investigators were also able to demonstrate the serological uniqueness of the virus in these cell lines; it appeared not to be related to one of the other human herpesviruses: herpes simplex virus (HSV), varicella zoster virus (VZV) or cytomegalovirus (CMV).

An unexpected observation led to the discovery of the causal relationship of EBV and infectious mononucleosis (IM). A laboratory technician whose serum served as an antibody negative control in the immunofluorescence test appeared to have undergone a seroconversion after having suffered

from a heterophile antibody positive IM. Later on retrospective and prospective studies confirmed the initial observation and it is now generally accepted that EBV causes this disease.

EBV can be isolated from throat washings during acute phase illness. Heterophile antibody negative IM can be caused by EBV also. In many patients this disease is caused by CMV or adenoviruses.

The first EBV-harbouring lymphoblastoid cell lines were established from biopsy cells of Burkitt's tumors, but now it is clear that the same can be done from any seropositive individual by culturing peripheral leukocytes, spleen cells or lymphnodes. These cell lines can be divided in 2 subgroups on the basis of presence (1) or absence (2) of viral antigens:

1) producer cell lines showing a small number of positive cells by immunofluorescence; these cells contain viral particles as detected by electron microscopy,

2) non-producer cell lines showing no positive cells by immunofluorescence and electron microscopy; however, these cells harbour the viral genome in multiple genome equivalents as demonstrated by nucleic acid hybridization.

As will be discussed later, it recently became possible to demonstrate the presence of an EBV-related nuclear antigen by anti-complement immunofluorescence in these non-producer lines (Chapter I of this thesis).

Before discussing the relationship between EBV and diseases other than IM, it is important to describe the four different *EBV-induced antigens*, that can be distinguished on the basis of conventional and anti-complement immunofluorescence.

1. Viral capsid antigen (VCA)

This one has been discovered first and is present only in producer cells. The antigen can be visualized on fixed cells by both immunofluorescence and anti-complement immunofluorescence. Its presence is correlated with synthesis of viral particles and is localized in the nucleus as well as in the cytoplasm.

2. Early antigen (EA)

This antigen can be demonstrated also in fixed cells by both methods. EA has two components: R(estricted), localized in the cytoplasm only and D(iffuse), localized both in cytoplasm and nucleus. EA is present in producer cells before induction of viral synthesis. It can be induced either by superinfection of non-producer cells with EBV and successive treatment with cytosin arabinoside (to prevent DNA synthesis), or by treatment of non-producer cells with the inducing agent 5-iododeoxyuridine (IUdR). Like VCA positive cells EA positive cells have entered the lytic cycle and ultimately die (Chapter III of this thesis).

3. Membrane antigen (MA)

This antigen also exhibits two components, early membrane antigen (EMA), produced early in the viral cycle and late membrane antigen (LMA), produced late in the viral cycle. MA can be demonstrated by direct

or indirect membrane immunofluorescence either on living biopsy or on cultured producer cells.

4. EBV-determined nuclear antigen (EBNA)

This antigen can be detected by anti-complement immunofluorescence only and it is localized in the nucleus of (a) all tumor cells of Burkitt's lymphoma and nasopharyngeal carcinoma, (b) a small percentage of the circulating lymphoblasts of IM patients and (c) in all EBV genome carrying lymphoblastoid cell lines. There is an absolute correlation between the presence of EBV-DNA, demonstrated by nucleic acid hybridization and the presence of EBNA. There is a strong correlation between EBNA and the EBV-determined complement fixing soluble (S) antigen. The latter antigen is present in the soluble fraction of all non-producer cell lines and is released from the cells by sonication and successive freezing and thawing.

As already mentioned above, EBV can be isolated from throat washings of IM patients. Transformation of cord blood lymphocytes, demonstrated by the appearance of EBNA and development of a continuously growing lymphoblastoid cell line, is the usual test to detect the virus. Another source of the virus is formed by producer cell lines. So far, no differences have been found between EBV isolates with a single exception: the P3HR-1 virus. This virus, isolated from a Burkitt's lymphoma producer cell line, is not able to transform cord blood lymphocytes, but induces EA production in non-producer cells.

The epidemiology of EBV resembles the epidemiology of other animal and human herpesviruses very much. Antibodies to VCA are found in a high percentage of newborns. These are from maternal origin and disappear within a few months. During the first decade of life the percentage of VCA antibody positive individuals increases very rapidly. The percentage varies from population to population and within these also with the socio-economic status. In certain African populations almost 100% of the young children at the age of 2-4 years have already been infected with EBV, while in western caucasian populations approximately 75% of the adolescents are seropositive. In the latter this percentage is even less in higher socio-economic classes.

Infection at childhood is not followed by any serious illness. Probably the only symptom is a mild upper respiratory tract infection. However, primary infection at adolescence is in 50-70% of the cases followed by a heterophile antibody positive IM. The severity of the symptoms can vary from a mild pharyngitis with some lymphnode involvement to a serious clinical illness with hepatosplenomegaly and massive lymphnode involvement.

After infection antibody patterns to the 4 different EBV antigens develop as follows:

1. VCA: IgM antibodies can be detected immediately after onset of the symptoms, but they disappear after several weeks. IgG antibodies can also

be detected shortly after onset of the disease and titers sometimes show a fourfold increase, if determined soon enough. Subsequently, antibody titers remain at a constant level throughout life.
2. EA: antibodies develop parallel to VCA antibodies. The titer does not remain constant and declines after several months, often to undetectable levels.
3. MA: antibodies are also found shortly after infection. Unfortunately, information on fluctuations in titer in post-IM patients is very limited. Healthy VCA positive individuals show low antibody titers to MA.
4. EBNA: antibodies develop after a latent period of 4-6 months. Once the titer is established, it remains constant throughout life.

The seriological diagnosis of IM is mostly based on the presence of heterophile antibodies. If these are absent, recent seroconversion for EBV can be demonstrated in several ways: (1) a fourfold increase in antibody titer to VCA and/or EA, (2) the presence of IgM antibodies to VCA, or (3) a combination of an antibody titer to VCA and EA and the absence of antibodies to EBNA. Especially the latter method can often be used and of great importance to discern from heterophile antibody negative IM, caused by other viruses. Recently, Klein and collaborators described the presence of EBNA positive cells in peripheral lymphocytes of EBV-induced IM, thus adding another diagnostic method.

After the discovery of EBV in human lymphoblastoid cell lines originating from Burkitt's tumors, its association with this and other malignancies could be further established. Evidence for a causal relationship is strongest with Burkitt's lymphoma (BL) itself and with nasopharyngeal carcinoma (NPC). Furthermore, some association is found with Hodgkin's disease (HD) and chronic lymphocytic leukemia (CLL). Also some nonmalignant diseases like sarcoidosis, systemic lupus erythematodes and lepromatous leprosy have been associated with EBV. The association of all these diseases with EBV, except BL and NPC, is mainly based on sero-epidemiologic studies, in which was found that mean antibody titers to EBV antigens are higher in patients than in age and sex matched controls. For BL and NPC evidence for a causal role of EBV is much stronger, although still circumstantial:
1. All patients with African BL and with NPC have antibodies to EBV antigens. The titers are often very high and show some disease-related fluctuations. In NPC, for instance, long term survivors show a clear decline of antibodies to EA, reaching normal levels, whereas a relapse of the tumor is preceded by a raise in antibody titer to EA. Antibody titers to MA in BL patients show a similar pattern. The addition "African" to BL is important to bear in mind, because the majority of Burkitt's lymphomas outside this continent is serologically not comparable. In case of NPC such a difference could not be found, although in this case genetic factors seem to influence the disease rates. Thus, some ethnic Chinese groups show a great incidence of NPC.

2. EBV is present in the tumor cells itself, *i.e.* the lymphoblastoid cell in BL and the carcinoma cell in NPC.
3. EBV is capable to transform lymphocytes *in vitro*, resulting in continuously growing immortalized lymphoblastoid cell lines.
4. EBV is capable of inducing malignant transformation *in vivo* in certain subhuman primates like marmosets and owl monkeys. Intravenous infection with EBV or with autologous transformed lymphocytes give rise to a malignant lymphoma, an IM-like disease, or no disease at all. These animals develop antibodies to EBV antigens and EBV can be isolated again. It remains to be established, whether intranasal or oral infection with EBV results in an epithelial cancer comparable with NPC.

One of the problems that remains to be solved, is the question, whether in some cases a ubiquitous virus like EBV can be causally related to malignancies, while usually a self-limiting disease or no disease at all is the result. Two theories are currently en vogue: (1) several subtypes of the virus exist and only a few are capable of transformation, or (2) genetically determined immune potential plays a major part in the resistance to the virus.

5. *Herpes simplex virus (HSV)*

In 1873 herpes simplex was shown to be infectious by human inoculation. At the turn of the century the viral nature of herpes simplex was established, but not until recently it has become apparent that isolates of this virus can be separated in 2 subgroups: type 1 and type 2. Herpes simplex virus type 1 (HSV-1) primarily causes infections mostly above the diaphragm by direct contact (*e.g.* kissing). Herpes simplex virus type 2 (HSV-2) produces infections mostly below the diaphragm and for infection a more intimate contact is necessary (*e.g.* sexual). The following criteria are used in order to differentiate between the types: (1) site of isolation and route of transmission, (2) giant cell formation and plaque size in tissue culture, (3) pocksize on the chorioallantois membrane of the fertilized chicken egg, (4) antigenic differences, (5) neurotropism following infection of mice and (6) nucleic acid hybridization. There is also a definite difference in temperature sensitivity.

Primary infection with HSV-1 generally occurs in childhood and can be followed by a gingivostomatitis, from extremely mild to very severe. Antibodies are demonstrable throughout life and at very constant levels, indicative for the persistent nature of the infection. Only a small minority of viral carriers show the typical herpes labialis. This is a recurrent outburst of oral blisters, which can be provoked by all sorts of aspecific stimuli. All patients have viral neutralizing antibodies and the titer of these remains unchanged in spite of the presence of infectious virus. The persistant nature of HSV-1 is demonstrated by its presence in the fifth cranial nerve. It can be shown in

the neuronal cells of the Gasserian ganglion by 2 different methods, (1) *in situ* hybridization with viral DNA and (2) by explanting the ganglion for 24-48 hours in organ culture, resulting in the production of viral antigens and virus in the ganglioncells, as detected by immunofluorescence and the typical cytopathological effect (CPE) in susceptible tissue culture cells. In fact, every HSV-1 infection of the cornea, skin or mucous membrane is followed by a subsequent latent infection of the corresponding sensory ganglia. Other clinical manifestations of HSV-1 include primary herpetic dermatitis, eczema herpeticum (Kaposi), keratitis dendritica (eye lesion) and herpetic encephalitis.

Primary infection with HSV-2 is mostly a venereal disease, called herpes genitalis. An other clinical manifestation HSV-2 is neonatal herpes, an almost 100% fatal primary infection of the neonatus. The mode of transmission is probably via the infected birth canal. Like HSV-1, HSV-2 can be isolated from sensory ganglia, in this case the sacral ganglia. Although antigenic differences between the 2 types of herpes simplex viruses exist, simple methods to discriminate between naturally occurring antibodies to either type of the virus are not available. Immunofluorescence or complement fixation tests are not suitable and the neutralization test is laboreous, time consuming and difficult to interpret.

Based on neutralization tests, sero-epidemiologic studies tend to associate HSV-2 infection and cervical carcinoma. This would confirm the earlier observations that cervical carcinoma behaves like a venereal disease and that sexual promiscuity and age of the first sexual intercourse influence the incidence of this malignancy. The main serological facts are that the percentage of patients that show antibodies to HSV-2 is higher than that of matched controls, although not all patients are infected. Furthermore, the mean antibody titer to HSV-2 is significantly higher in patients than in controls. However, HSV-2 specific nucleic acids and antigens can not be found in tumor cells, as is the case for EBV. One group of writers has claimed the presence of viral DNA in a cervical carcinoma, but this could not be confirmed by other investigators.

HSV-1 has recently been associated with squamous cell carcinoma of the head and neck region. This is again based on the same sort of evidence as used for HSV-2 and cervical carcinoma. Up till now no trace of the virus could be demonstrated in these tumors.

Experiments in laboratory animals clearly show the oncogenic potential of HSV-1 and HSV-2. Both viruses, after UV irradiation, are capable of transforming hamster and mouse cells. These transformed cells grow out as malignant tumors in the syngeneic host. These animals develop specific antibodies to HSV-1 or HSV-2. Viral antigens can be demonstrated in the tumor cells. A puzzling, probably pure technical problem is the impossibility to demonstrate viral DNA in these tumor cells with the most sensitive nucleic acid hybridization methods. This should be kept in mind when no viral DNA can be found in human tumor cells.

HSV-1 and HSV-2 are still very interesting candidates as possible human oncogenic viruses, but evidence is very circumstantial and much less impressive than in the case of EBV.

6. *Cytomegalovirus (CMV)*

Human cytomegalovirus is a highly species-specific herpesvirus, capable of infecting without any symptom or with severe and fatal disease. As discussed earlier, this is a common characteristic of all herpesviruses. Infection with CMV leads to distinctly enlarged cells, *i.e.* cytomegaly, containing intranuclear and cytoplasmic inclusions. These inclusions contain CMV particles. The virus can be isolated from throat swabs and urine specimens and cultured on human fibroblasts, resulting in a characteristic CPE. Recently, CMV could be isolated also from polymorphonuclear lymphocytes and from semen (primarily extracellular). A recent infection by CMV can be demonstrated by a wide variety of serological methods. Virus neutralization, complement fixation and immunofluorescence are widely used to demonstrate the virus and naturally occurring antibodies. Either a fourfold increase in antibody titer or the presence of IgM antibodies is indicative for recent infection. A remarkable exception is the persisting presence of IgM antibodies in congenital CMV infection. Like in EBV, CMV induces a membrane antigen, a late viral antigen and an early antigen. An equivalent antigen to EBNA has not yet been discovered.

Diseases caused by CMV are the following:

1) Cytomegalic Inclusion Disease of Infancy: this is a result of congenital infection, which may also lead to spontaneous abortion.

2) CMV mononucleosis (heterophile antibody negative).

3) Postperfusion syndrome: a CMV mononucleosis caused by infection via bloodtransfusion with fresh blood (less than 24 hours old). Disease-related changes in antibody titers to the 3 CMV antigens have been described.

Evidence for a possible oncogenic potential of CMV in humans is limited:

1) After UV irradiation of the virus, CMV is capable of transforming mouse and hamster fibroblasts. These transformed cells behave like malignant tumor cells (mostly sarcomas) when injected in newborn animals. CMV antigens can be demonstrated in the tumor cells and antibodies to CMV develop in these animals.

2) CMV is capable of causing a self-limiting lymphoproliferative disease, quite similar to EBV-induced IM.

3) There is some evidence on the presence of CMV in tumor cells of patients with Kaposi's sarcoma.

4) Recently, a genital isolate of CMV appeared to be capable to transform human embryonic fibroblasts and these transformed cells produce progres-

sive growing tumors in wheanling athymic nude mice.

With respect to the first item, there is a confusing observation about the expected presence of CMV-DNA in these transformed cells. It seems impossible to demonstrate this with nucleic acid hybridization, even under conditions which permit the demonstration of 0.2 CMV genome equivalent per cell. This would imply that less than 20% of the viral genome may be involved in the process of malignant transformation and is sufficient to code for (some) viral antigens. This situation is very similar to that for HSV-1 and HSV-2. This also implies that the negative attempts to demonstrate the presence of CMV-DNA in all sorts of human tumors by nucleic acid hybridization are not yet conclusive about the possible oncogenicity of CMV.

One should bear in mind that the frequent isolation of CMV from cancerous patients can be the result of either spontaneous or drug-induced immunosuppression, *e.g.* such as in renal transplant patients.

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INTRODUCTION TO THE ARTICLES

The aim of the experimental work presented in the five papers of this thesis was to improve known immunological techniques for the demonstration of antibodies to and antigens of human herpesviruses. The most important technique used was conventional immunofluorescence, either direct or indirect. This test could be refined by using a mixture of infected and uninfected cells and by counterstaining the cells with Evans' blue. For all four herpesviruses, *i.e.* herpes simplex virus (HSV), varicella-zoster virus (VZV), cytomegalovirus (CMV) and Epstein-Barr virus (EBV) essentially the same technique could be used, allowing quick, accurate and comparable antibody titer determinations. When these tests were developed, Reedman and Klein started to use anti-complement immunofluorescence, with which they were able to demonstrate the EBV-determined nuclear antigen (EBNA). This antigen, for as yet unknown reasons, cannot be demonstrated by conventional immunofluorescence. Application of the same refinements to this method and the use of mixtures of EBNA positive and negative cells, resulted in (1) higher titers and more accuracy in titrations, (2) thereby the possibility of using the absorption procedure in a semi-quantitative, more sensitive way and (3) finally the simultaneous detection of the possible presence of anti-nuclear factor (ANF). These methods are described in Chapters 1 and 4 of this thesis.

Up till now, the immunofluorescence absorption (IFA) and anti-complement immunofluorescence absorption (ACIFA) tests are the only usefull semi-quantitative tests for the various EBV antigens except the membrane antigen (MA).

Since it is not possible to quantitate the MA on fixed cells, the radioiodine elution (RIE) test was used for the relative quantification of MA on living cells. This led to the unexpected finding, that in this test not only MA is quantitated, but also intracellular antigens VCA and EA and probably EBNA as well (Chapter 2 of this thesis). Therefore, a method was developed to show, by direct immunofluorescence, that "living" cells are indeed accessible to specific antibodies. Again, the use of the counterstaining dye Evans' blue appeared to be indispensable (Chapter 3 of this thesis). The practical use of the indirect and anti-complement immunofluorescence tests for sero-epidemiological purposes has been shown in the Chapters 4 and 5.

Each article is preceeded by a summary, which is not repeated here.

CHAPTER 1

Immunofluorescence and anti-complement immunofluorescence absorption tests for quantitation of Epstein-Barr virus-associated antigens.

Int. J. Cancer: **15**, 566-571 (1975)

IMMUNOFLUORESCENCE AND ANTI-COMPLEMENT IMMUNOFLUORESCENCE ABSORPTION TESTS FOR QUANTITATION OF EPSTEIN-BARR VIRUS-ASSOCIATED ANTIGENS

by

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Immunofluorescence absorption methods are described which permit quantitative estimation and differentiation of Epstein-Barr virus (EBV)-associated antigens (virus capsid antigen, VCA, early antigen, EA and EBV-determined nuclear antigen, EBNA) in cell extracts. EBNA was present in all cell lines (producer and non-producer) which carried the EBV-genome, while VCA and EA were present in producer lines only. All the antigens were absent from a lymphoid cell line (MOLT-4) which lacked the EBV-genome, as well as from leukemia cells from peripheral blood. The techniques demonstrated antigenic identity of the various antigens when prepared from different cell lines.

Complement fixation has hitherto been a useful method for titrating EBV-associated antigenic activity in extracts of lymphoblastoid cell lines (Pope *et al.*, 1969; Vonka *et al.*, 1970*a*, 1970*b*; Walters and Pope, 1971). This method has the disadvantage that it does not discriminate between the various antigens recognized in immunofluorescence tests, *viz.* VCA, EA, EBNA and membrane antigen (MA). All EBV-carrying lymphoblastoid cell lines produce EBNA (Reedman and Klein, 1973), while only some lines, designated producer lines, synthetize the other antigens. While VCA and EA can be detected in fixed cells by direct or indirect immunofluorescence, a more sensitive test, anti-complement immunofluorescence, is required for EBNA. It is likely that EBNA is the same as the EBV-associated soluble complement-fixing antigen known to be present in extracts of producer and non-producer cells. It is equally likely that VCA, EA and MA contribute to the complement-fixing activity of extracts of producer cell lines. In this paper, immunofluorescence absorption techniques which have been useful for the quantitation of RNA-tumour virus antigens (Hilgers *et al.*, 1972) were adapted to the estimation of VCA, EA and EBNA in extracts of lymphoblastoid cells.

MATERIAL AND METHODS

Cell lines and antisera

The cell lines used are listed in Table I. They were propagated as stationary cultures in RPMI 1640 medium with 15% foetal calf serum.

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Reprint requests should be sent to the Librarian, Queensland Institute of Medical Research, Herston Road, Herston, Q 4006, Australia.

P3HR1 cells with a high percentage of VCA-producing cells were used as target cells for the titration of VCA in cell extracts. An FITC-conjugated serum (F-Simiya) with antibody to VCA but very low levels of antibody to EA was used as the reference serum in absorption tests.

Daudi cells were infected with EBV harvested from P3HR1 cells by Dr. Alice Lindahl. The Daudi cells were preincubated with virus for 1 h at 37° C, washed and incubated in fresh medium for 48 h. Cytosine arabinoside (5 μg/ml) was included in the medium to inhibit DNA synthesis, thus allowing EA but not VCA to develop in the superinfected cells. These cells were used as targets for the titration of EA in cell extracts. An FITC-conjugated serum (F-Nathan) with a high level of antibody to EA was the reference serum for absorption tests.

Raji cells were the targets for EBNA tests. Raji is a non-producer line, so that VCA and EA fluorescence did not interfere in these tests. A serum (OO) with a high titre of antibody to EBNA was the reference serum for absorption experiments.

Cell extracts

The extraction procedure was that developed earlier for complement fixation studies (Reedman *et al.*, 1972). Cells were harvested by centrifugation, washed three times in balanced salt solution (BSS) and resuspended in BSS. They were frozen and thawed, sonicated intermittently 8-10 times and centrifuged at $10{,}000 \times g$ for 5 min. The supernatants were used as antigens for absorption. These extracts would be expected to contain a proportion of the various membrane-bound and soluble antigens of the cells, but determination of the efficiency of the extraction procedure was outside the scope of this paper.

Preparation of smears

Slides with eight or 10 separate wells were prepared by coating standard microscope slides with a hydrophobic teflon film (Hilflon, Hylin Works, Enfield, Middlesex, England) or bought commercially (Cooke slides, Cooke Instruments AG, Zolliken, Switzerland). Target cells were harvested and washed three times in BSS. For VCA and EA tests, cells were resuspended at a concentration of 1×10^6 cells/ml in saline and 30-50 μl drops placed in each well of the multi-well slides and allowed to dry overnight. For EBNA tests, 5 μl drops of a concentrated cell suspension (approximately 10×10^6 cells/ml) were placed in each well, quickly spread over the whole well and dried quickly. All slides were fixed in acetone for 10 min and stored at −20° C or −90° C until use. Before the immunofluorescence tests were started, slides were warmed to room temperature, washed in distilled water to remove salts, and dried.

Titration of reference sera

Reference sera (F-Simiya, F-Nathan and OO) were titrated accurately before being used in absorption tests. In each case, two-fold dilutions of the appropriate serum were prepared in BSS. All incubations were performed at 37° C for 45 or 60 min in a humid chamber. As a final step in each procedure, slides were counterstained with Evans' blue (0.001% in distilled water) for 5 min, rinsed in distilled water, dried and mounted in BSS-glycerol (1:1). They were examined in a Leitz Ortholux microscope equipped with a vertical Ploem-type illuminator at oil immersion (×54 or ×100).

Direct immunofluorescence. Smears of target cells were incubated with dilutions of F-Simiya or F-Nathan, washed in two changes of BSS for 5 min each, rinsed and counterstained. The brightly staining EA or VCA-positive cells were readily distinguished against a background of unstained cells. The endpoint was read as the final dilution of serum still giving positive specific fluorescence when compared with control smears incubated with BSS only.

Anti-complement immunofluorescence. In these tests, an EBV-negative human serum diluted 1:10 in BSS was used as a source of complement. Undiluted serum was stored in small aliquots at −90° C. Prolonged washing of the slides was important.

Smears of target cells were incubated with dilutions of reference serum (OO), washed in BSS for 30 min, rinsed in distilled water and dried. Complement was added to each well and the slides were incubated, washed for 30 min and dried. They were stained with fluorescein-conjugated anti-human complement (anti β_{1C}/β_{1A}, Hyland Laboratories, Los Angeles, Calif.,

TABLE I

TITRATION OF VCA, EA AND EBNA IN EXTRACTS OF EBV-CARRYING AND CONTROL CELLS

Cell line	Reference	Origin	Type of cells[1]	EBV-status	Cell concentration (cells/ml)	Antigen titres (reciprocal)		
						VCA	EA	EBNA
QIMR-WIL	Pope (1968)	Leukaemia	B-LCL	Producer cell line	10^9	8	24	64
Raji	Pulvertaft, 1965; Epstein *et al.*, 1966	Burkitt's lymphoma	B-LCL	Non-producer cell line	10^9	<1	<1	32
P3HR1	Hinuma and Grace, 1967	Burkitt's lymphoma	B-LCL	Subline 1: high level of VCA-, EA-positive cells	10^9	16	32	24
				Subline 2: intermediate level of VCA-, EA- positive cells	10^9	4	8	16
				Subline 3: low level of VCA-, EA-positive cells	10^9	1	6	32
Daudi	Klein, E., *et al.*, 1968	Burkitt's lymphoma	B-LCL	Producer cell line	10^9	8	16	8
Molt-4	Minowada *et al.*, 1972	Leukaemia	T-LCL	EBV-negative	5×10^8	<1	<1	<1
Leukaemia cells		Leukocytes from peripheral blood		EBNA-negative	10^9	<1	<1	<1

[1] B-LCL: lymphoid cell line with B-cell characteristics.
T-LCL: lymphoid cell line with T-cell characteristics.

USA), washed for 30 min, rinsed and counterstained. The endpoint was read as the final dilution of serum giving detectable nuclear fluorescence when compared with a control smear stained with BSS, complement and conjugate only.

Immunofluorescence absorption tests

Absorption of the direct immunofluorescence of F-Simiya and F-Nathan was used for the titration of VCA and EA, respectively, in cell extracts. Anti-complement immunofluorescence absorption was used to titrate EBNA in cell extracts, with OO as reference serum.

In each case, reference sera were titrated as described above and diluted to contain four units of antibody (*i.e.* two steps before the endpoint). Two-fold dilutions of cell extract were incubated at 4° C overnight with an equal volume of diluted reference serum. Drops of the serum-antigen mixtures were placed on target cells on the multi-well slides, and the direct immunofluorescence or the anti-complement immunofluorescence procedure followed as described above for the titration of reference sera.

In each case, titres were read as the final dilution of antigen extract causing detectable inhibition of the immunofluorescence reaction, when compared with control smears stained with diluted reference serum.

The reproducibility of the absorption tests was found, in practice, to depend on accurate titration and dilution of the reference sera. Controls were included to check that the dilutions of sera used contained four units of antibody. Each antigen was titrated in duplicate and the mean titre determined.

RESULTS AND DISCUSSION

Extracts were prepared from several lymphoblastoid cell lines and control cells and VCA, EA and EBNA levels measured in each (Table I).

The results shown are the mean titres for each antigen tested in duplicate and on at least two occasions. Several relevant conclusions can be made from this Table. All the EBV-carrying lymphoblastoid cell lines, irrespective of their producer or non-producer status, contained EBNA. Only producer cell lines (QIMR-WIL, P3HR1 and Daudi) contained EA and VCA. Within this group, subline 3 of P3HR1, with a low level of VCA and EA-positive cells by immunofluorescence, contained almost no detectable VCA and a much lower level of EA than did the extract of subline 1. Subline 2, with an intermediate number of EA and VCA-positive cells by immunofluorescence, gave intermediate levels of EA and VCA. There was no significant difference between the levels of EBNA in the three extracts. Furthermore, the absorption tests indicate that VCA, EA and EBNA from various sources are antigenically closely related. It is still possible that more sensitive tests would demonstrate minor antigenic differences from one cell line to another.

Absorbing activity was not found in cells which did not carry the EBV-genome. Molt-4 is an EBV-negative lymphoid cell line with T-cell characteristics (Minowada *et al.*, 1972; Jondal and Klein, 1973), as compared with the EBV-positive cells which have B-cell characteristics (Jondal and Klein, 1973). Molt-4 extracts were non-reactive in VCA, EA and EBNA absorption tests, as was an extract of leukaemic leukocytes taken from peripheral blood (Table I).

It was not considered valid to compare relative levels of VCA, EA and EBNA in individual cell extracts as different reference antisera, target cells and immunofluorescence techniques were involved. Furthermore, immunofluorescence absorption is dependent upon the affinity of binding as well as on the actual amounts of antigens, and at present the former cannot be determined. However, the results are in keeping with previous observations that EBNA is difficult to detect because of its relatively low level in the majority of cells, while VCA and EA are concentrated in a small proportion of cells and are readily detected by direct immunofluorescence.

With low dilutions of antigen in the anti-complement immunofluorescence absorption test fluorescence was often seen around the periphery of Raji cells. This effect disappeared as the endpoint approached and did not interfere with the recognition of the distinctive EBNA staining. Raji cells have been reported to carry receptors for Fc and complement (Theophilopoulos *et al.*, 1974) and it is possible that attachment of immune complexes gave rise to the fluorescence on the surface of Raji cells. However, it is not known whether these receptors would have survived the fixation process.

Daudi (a producer cell line) and Raji (a non-producer cell line) were incubated with 5-iododeoxyuridine (IUDR) as indicated in Table II. Significant increases in EA levels of the cell extracts were found in both cases. This is consistent with published reports of the induction of EA by treatment of cells with IUDR (Sugawara *et al.*, 1972; Klein and Dombos, 1973). There was no significant increase in the levels of EBNA in the cell lines, although a very small increase may not have been detectable in this system. A recent report suggested that there was an increased level of a nuclear antigen (possibly EBNA) in non-producer cells treated with IUDR (Sugawara and Osato, 1973).

Previously, complement fixation was used for the quantitation of EBV-associated antigens in producer and non-producer lymphoblastoid cell lines. The absorption techniques described here have the advantage that they differentiate between the antigens VCA, EA and EBNA, all of which probably contribute to the complement fixation activity. This study was not extended to the measurement of MA which would have been complicated by the requirement for live target cells. The techniques are useful for following the individual antigens during purification procedures, as well as for demonstrating antigenic identity in cell extracts. In this respect, it was shown that an EBV-related nuclear antigen in Burkitt lymphoma biopsies was at least closely related, if not identical, to EBNA in the lymphoblastoid cell lines (Reedman *et al.*, 1974).

TABLE II

EFFECT OF IUDR TREATMENT ON RAJI AND DAUDI CELL LINES

Cells [1]	Antigen titres		
	VCA	EA	EBNA
Raji, no IUDR	<1	<1	24
Raji+IUDR	<1	4	32
Daudi, no IUDR	NT [2]	8	4
Daudi+IUDR	NT	64	6

[1] Cells were incubated with or without IUDR (100 μg/ml) for 48 h, harvested and resuspended at 5.0×10^6 cells/ml.
NT = not tested.

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TESTS D'ABSORPTION DE L'IMMUNOFLUORESCENCE DIRECTE ET DE L'IMMUNOFLUORESCENCE ANTI-COMPLÉMENT POUR LA DÉTERMINATION QUANTITATIVE DES ANTIGÈNES ASSOCIÉS AU VIRUS D'EPSTEIN-BARR

Les auteurs décrivent des méthodes d'absorption de l'immunofluorescence qui permettent une estimation quantitative et une différenciation des antigènes associés au virus d'Epstein-Barr (antigène des capsides virales (VCA), antigène précoce (EA) et antigène nucléaire déterminé par l'EBV (EBNA)) dans les extraits cellulaires. L'EBNA était présent dans toutes les lignées cellulaires (productrices et non-productrices) qui portaient le génome de l'EBV, alors que le VCA et l'EA n'apparaissaient que dans les lignées productrices. Aucun de ces antigènes n'a été décelé dans une lignée lymphoïde (MOLT-4) qui ne portait pas le génome de l'EBV, ainsi que dans les cellules leucémiques du sang périphérique. Ces techniques ont mis en évidence une identité antigénique des divers antigènes préparés à partir de lignées différentes.

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CHAPTER 2

Radioiodine-labeled antibody elution (RIE) for detection of EBV-determined antigens: competition for specific labeled antibodies using suspended antigens.

Radioiodine-Labeled Antibody Elution (RIE) for Detection of EBV-Determined Antigens: Competition for Specific Labeled Antibodies Using Suspended Antigens[1]

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Antigens determined by Epstein Barr virus (EBV) have been detected and quantitated in human lymphoblastoid cell culture lines by specific radioiodine-labeled antibody elution (RIE). The peak of specific elutable antibody has been shown to quantitatively increase with increasing doses of EBV 2–3 days after superinfection of susceptible lines. Increasing antibody concentrations on constant cell numbers produced a linear increase in elutable antibody binding specifically to the producer cells. Increasing cell numbers in the presence of a constant antibody concentration, likewise, produced a graded increase in the specific antibody eluted from the producer cells. Antigen suspensions prepared from frozen and thawed cells were used to compete with the antigens on the target cells for the antigen-combining sites of the labeled antibodies. Experiments were performed using suspensions from cells containing all the EBV-determined antigens for competition as well as limiting the competition to early antigen (EA).

Continuously growing lymphoblastoid cell lines of human origin contain the Epstein Barr virus (EBV) genome and possess antigens determined by EBV (1). Different cell lines are categorized as producers or nonproducers by the amount of the EBV-determined antigens they possess. These antigens include: membrane antigen (MA) (2,3), viral capsid antigen (VCA) (4–6), and early antigen (EA), so named because it appears as an early intracellular viral product after superinfection of a nonproducer line with EBV (7). EA has been subdivided into restricted (R) and diffuse (D) subtypes with different antigenic specificities (8). MA, EA, and VCA are not present in detectable levels in the nonproducer lines, although all the lines which have been studied have been found to contain the EBV genome by DNA/DNA (9) and cRNA/DNA (10,11) hybridization studies. The one antigen which appears to be present in all these lines, including the nonproducers, is the recently defined EBV nuclear antigen (EBNA), detectable in 85–95% of the nuclei by anticomplement immunofluorescence (12). Some of the lines can be induced to produce the virally determined EA by exposure to 5-iododeoxyuridine (IUDR) (13) or 5-bromodeoxyuridine (BUDR) (14,15). Superinfection of some lines with EBV will result in the production of MA and VCA as well as EA (13). If the superinfection is carried out in the presence of the DNA inhibitor cytosine arabinoside (Ara C) only MA and EA will be produced (16).

Antibodies to these antigens appear regularly, often at high titers, in the sera of patients with Burkitt's lymphoma (BL) (17,18) nasopharyngeal carcinoma (19,20) and infectious mononucleosis (21–23). Antibodies from some patients react with MA, EA, and VCA, while antibodies from others react only with MA and VCA. Sera from a relatively small "discordant" group react with only VCA and EA (17,24). Sera defined with respect to these reactivities provide the reagents for detection of the various antigens determined by EBV.

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MATERIALS AND METHODS

Tissue-culture lines. The cell lines used in these experiments and their characteristics are listed in Table 1. They were maintained in suspension culture with RPMI supplemented with 15% fetal calf serum (FCS), 100 μg/ml penicillin, and 50 μg/ml streptomycin, incubated at 37°C in a 5% CO_2 atmosphere. Producer cell lines used were tested just prior to feeding which was performed every 3 days.

TABLE 1
In Vitro HUMAN CELL CULTURE LINES

Designation	Source	Induction/superinfection	EBV Genome	EBNA	MA	VCA	EA	Category
Maku	BL (28)		+	+	+	+	+	Producer
Namalwa	BL (29, 30)		+	+	−	−	−	Nonproducer
Raji	BL (31, 32)	Untreated	+	+	−	−	−	Nonproducer
		IUDR			−	−	+	
		EBV superinfection			+	(+)	++	
Daudi	BL (33)	Untreated	+	+	(+)	(+)	(+)	Low-level producer
		IUDR			−	−	++	
		EBV superinfection			+	+	++	
Simpson	Myeloma (34)		−	−	−	−	−	Non-EBV line
698	Lymphoma[a] (non-Burkitt)		−	−	−	−	−	Non-EBV line

EBNA is demonstrable in 85–95% of the cells which possess the EBV genome. + for MA indicates that 25–90% of the cells are positive. + for VCA indicates 0.5–2% positive cells. EA is present in 1–4% of the producer cells, induction or superinfection of Raji or Daudi results in much higher levels of EA (as high as 10–40%). Parentheses indicate low levels.

[a] This line was received from Dr. Kenneth Nilsson, Uppsala, Sweden.

Labeled immunoglobulins. IgG fractions of the following sera were used: (a) Abwao, reactive with MA, EA, and VCA; (b) Nathan, reactive with only EA and VCA. IgG in 100-μg samples was labeled with 1 mCi of ^{125}I by the chloramine T method (35) as previously described (25, 26). The labeled antibody in a total volume of 2.6 ml 0.15 *M* phosphate buffer, pH 7.4, with 5% FCS was stored at −4°C in lead shielding and used at the dilutions indicated in the results.

Virus. EBV was prepared from the supernatant of P3HR-1 cells (36) by the polyethylene glycol concentration method as previously described (37) and stored at −90°C prior to use. Superinfection was carried out by incubating 10^6 cells in 1 ml of various dilutions of virus for 1 hr, followed by a 4-fold increase in the volume with medium and subsequent incubation for 2–4 days. Virus preparations used in these experiments induced 10–20% EA-positive cells in the Raji line at a dilution of 1:20 48 hr after infection. In some experiments cytosine arabinoside (Ara C) was added at a concentration of 20 μg/ml to inhibit DNA synthesis and VCA production (16).

Induction. Raji and Daudi cells were exposed to 25 μg/ml of IUDR and the cells were harvested 2–4 days later in parallel with untreated control cells.

Radioiodine-labeled antibody elution (RIE). Experiments were performed in triplicate with tubes rinsed with undiluted FCS using 10^6 test or control target cells per tube, or graded numbers of test and control cells when cell titrations were performed. The cells were incubated for 1 hr at 4°C with 0.05 ml of the labeled reagent antibody followed by four or five sequential washes with 1 ml of cold medium containing 10% FCS. The cells were then incubated with 1 ml of pH 2.8, 0.1 *M* glycine–HCl buffer for 30 min at 37°C to elute specifically bound antibody, and the pellet was washed two more times with medium. The sequential washings and acid eluate were counted in a Nukab gamma counter. The mean counts per minute of the triplicate washings in each wash and elution were calculated and the counts per minute in the control cell washings and elution were subtracted from the matched counts per minute in the test cell washings and elution in order to show the specific amount of elutable antibody binding to the test cells. The specific elutable antibody binding expressed as counts per minute gives a relative quantitation within a single experiment. Only experiments using the same antibody from a single labeling can be quantitatively compared. These are indicated in the results.

Competition with suspended antigens. Cells ($4–10 \times 10^7$) were pelleted and frozen and thawed twice. The pellet was resuspended in 0.5 ml of medium and sedimentable debris was removed by centrifugation at 5000*g*. The supernatant was saved and used as suspended antigen to compete with the target cells for the antigen-combining sites of the labeled antibodies. These preparations were from cells possessing EBV-determined antigens or from the same number of various control cells as indicated in the results. Hereafter, these suspensions will be referred to as antigen extracts or control extracts, respectively. The antigen extracts (0.05 ml) were added to the test cells at the same time as the labeled antibody. Reduction in the acid elution peak compared to the peak from cells receiving only labeled antibody alone

TABLE 2

RADIOIODINE-LABELED ANTIBODY ELUTION TEST OF ^{125}I-LABELED Abwao (1:2) ON 10^6 RAJI CELLS SUPERINFECTED 4 DAYS PREVIOUSLY WITH EBV (1:10)[a]

Wash no.	cpm ± SD		Test-control	*t*
	Test	Control		
3	4256 ± 530	3864 ± 744	392	0.74
4	1220 ± 463	683 ± 209	538	1.83
5	485 ± 138	339 ± 112	147	1.43
pH 2.8	1420 ± 54	427 ± 26	993	28.7
7	291 ± 55	148 ± 12	143	4.35
8	152 ± 23	100 ± 2	52	3.97

[a] Control cells were untreated Raji. Mean values are from triplicate samples. *t* values shown were calculated using a Student's *t* test.

or with control extracts indicated successful competition by the suspended antigen preparation for the labeled antibody.

RESULTS

Table 2 shows the results of one experiment in detail to illustrate how the data were evaluated. In this experiment, Raji cells were superinfected with EBV 4 days prior to testing. There were no significant differences between the supernatants from the control and test cells until the specific antibody (Abwao IgG) was eluted with acid. The amount of specific elutable antibody bound to the EBV-superinfected Raji cells can be expressed by subtracting the counts per minute in the control cell supernatants as shown in Fig. 1. That this peak represents active antigen synthesis by the superinfected cells and not simply adsorbed virus on the surface of the cells was demonstrated by performing the same test within 1 hr after the addition of the same dilution of virus. There was no binding of elutable antibody to the cells at this time. The

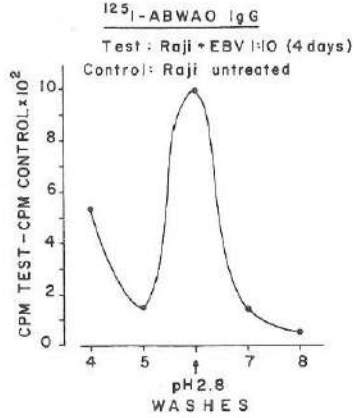

FIG. 1. Specific acid elution peak of antibody binding to EBV-superinfected Raji cells. Data shown in Table 2, ^{125}I-Labeled Abwao IgG was used diluted 1:2.

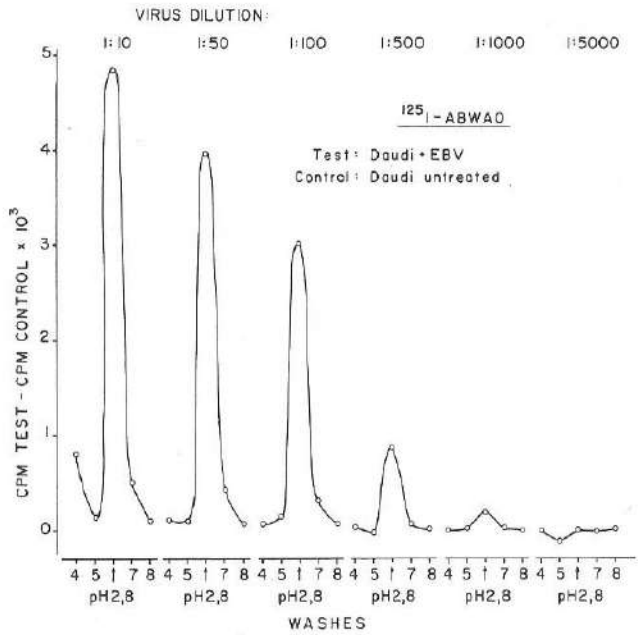

FIG. 2. Quantitation of antigen production in response to EBV superinfection of Daudi cells 3 days previously. Labeled Abwao IgG was used diluted 1:10.

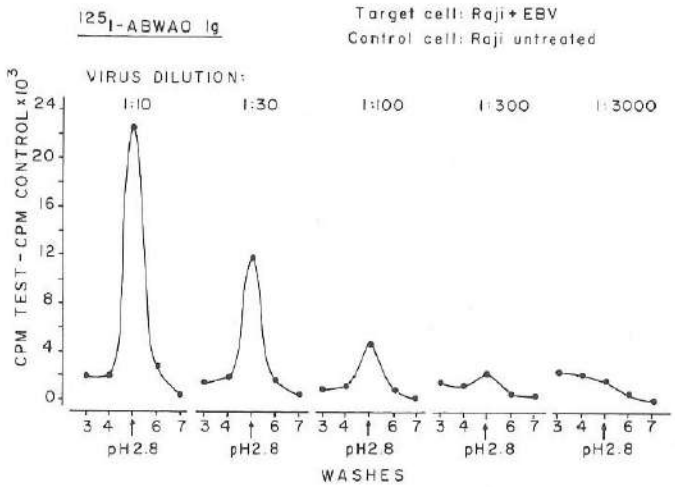

FIG. 3. Quantitation of antigen production in response to EBV superinfection of Raji cells 2 days previously. IgG concentration was the same as in Fig. 2.

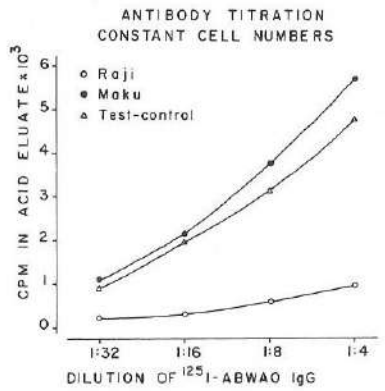

FIG. 4. Antibody titration with constant cell numbers: 10^6 test and control cells per tube. The points represent the mean counts per minute of triplicate tubes containing the acid eluate only. Test cells are Maku and control are Raji.

earliest antigen induction we have detected by this method is 2 days after superinfection (38).

In order to test the quantitative sensitivity of the RIE, Raji or Daudi cells were superinfected with EBV and tested for antigenicity with labeled Abwao IgG 2 and 3 days later. The results of these experiments are shown in Figs. 2 and 3. An increase in antigenicity could be detected with a virus dilution as high as 1:1000 with Daudi cells and 1:300 with Raji cells.

In order to further investigate the quantitative aspects of the test, titrations of producer cells with constant antibody (Abwao) concentrations and antibody titrations with constant cell numbers were performed. The latter experiment (Fig. 4) shows the counts per minute in the acid eluate from the test cells Maku and from the control cells Raji. By subtracting the counts per minute of control eluates from those of the test it can be seen that within this range of antibody concentrations on 10^6 cells there is a linear increase of specific elutable antibody binding to the Maku cells with increasing antibody concentrations.

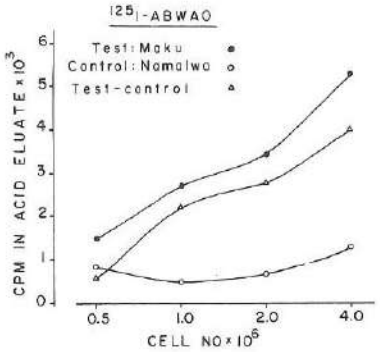

FIG. 5. Cell titration with constant antibody concentration (Abwao 1:16). Test cells are Maku and control cells are the nonproducer Namalwa.

Using a 1:16 dilution of this labeled antibody, the cell titration shown in Fig. 5 demonstrated an increase in specific antibody binding to the Maku cells as the cell number was increased.

Competition with suspended antigen. In the first of these experiments the antigen extract was prepared from Daudi cells which had been superinfected 4 days previously. As shown in Fig. 6 the antigen extract reduced the amount of specific elutable antibody binding to the Maku cells by one third (1000 cpm) compared to cells receiving antibody alone. It was also shown that the antigen extract added to the control target cells (Raji) produced no significant elutable antibody binding to these cells.

In order to demonstrate that this reduction in counts was due to specific antigen in the extract and not due to some nonspecific reduction in the antibody binding by other components in the preparation, a similar experiment was performed adding a control extract from the same number of non-EBV-carrying cells, in parallel with the antigen extract from the superinfected Daudi cells. In this experiment (Fig. 7) there was a reduction of 2500 cpm (34%) in elutable antibody binding to the superinfected Daudi target cells by the antigen extract compared to the elution peak from the cells receiving control extract from the same number of Simpson cells. The latter cells are from a myeloma line possessing no EBV-determined antigens.

In order to determine if this competition by suspended EBV antigens was quantitative, the antigen extract was diluted in a stepwise manner and tested in parallel with a control extract from the same number of cells. The competition experiment shown in Fig. 8 is with antigen equivalent to the extract of 4, 2, and 1×10^6 superinfected Daudi cells per tube and control extract from the same number of 698 cells (non-EBV-containing lymphoma cell line). The elution peak on the extreme left is the positive control with no competing extract. The control extract from the 698 cells produced only minor fluctuations in the

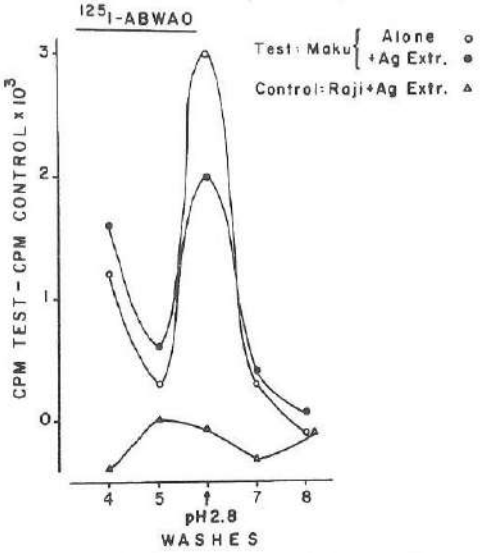

FIG. 6. Competition with suspended antigen for the labeled antibodies. Antigen extract was prepared from 10^6 Daudi cells which had been superinfected with EBV (1:40) 4 days previously. The extract (0.05 ml—equivalent to the extract from 10^7 cells) was added to the test cells (Maku) and control cells (Raji) at the same time as the labeled antibody.

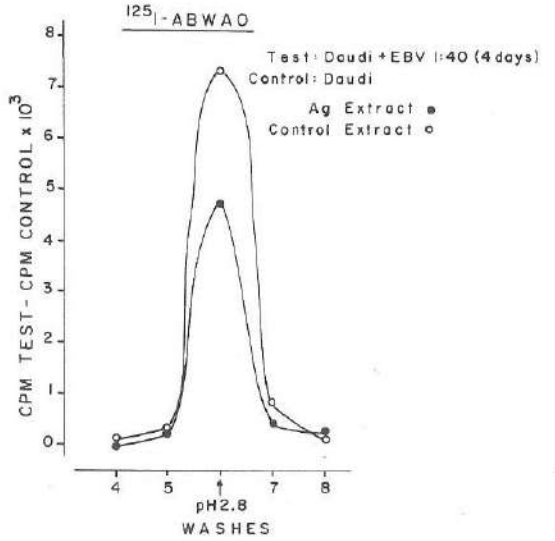

FIG. 7. Competition with suspended antigen from frozen and thawed superinfected Daudi cells (as in Fig. 6) compared to control extract from the same number of cells (10^7/tube) of the non-EBV line Simpson.

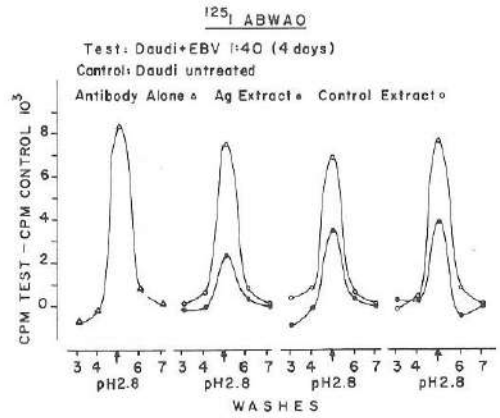

FIG. 8. Titration of competitive activity with specific antigen extract from superinfected Daudi cells (as in Fig. 6) compared to the extract from the same number of cells from the non-EBV 698 line. Competition from left to right represent extracts from 0 cells, 4×10^6, 2×10^6, and 10^6 cells.

quantity of specific elutable antibody binding to the superinfected Daudi target cells (8, 17, and 7% reduction in the elution peak compared to the cells with no competing extract). In contrast, the specific elutable antibody binding was decreased by 71, 58, and 53% with sequentially decreasing quantities of antigen extract.

In the preceding experiments the competition with suspended antigen gave no indication of which EBV-determined antigens were active in the reaction, since the Abwao antibody used reacts with all the antigens, and the targets as well as the cells used for the antigen extracts possessed all the EBV-determined antigens. In the following experiments, however, the competition was limited to EA. In the first of these EA competition experiments Daudi cells were superinfected with EBV in the presence of AraC 4 days prior to testing. The cells, thus treated, were MA and EA positive but VCA negative and were used as a source of extract as well as target cells. The labeled antibody used was the MA−, EA+, VCA+ Nathan IgG. The only antigen which could be detected, therefore, was EA. The addition of the antigen extract reduced the amount of elutable antibody binding to the test cells by 70% (> 19,000 cpm) as shown in Fig. 9. Also, no change in the negative elution profile was observed by the addition of the antigen extract to the control Raji cells. In a subsequent experiment Daudi cells were treated with IUDR 2 days prior to testing and used as a source of antigen extract and as targets. IUDR-treated Daudi cells have an increase in EA, but no VCA is produced. Control extract was prepared from the same number of 698 cells. There was a 63% reduction in the counts by the added extract from the IUDR-treated cells compared to the elution peaks from the test cells receiving control extract (Fig. 10). A similar experiment was performed using IUDR treated Raji cells as a source of antigen extract and untreated Raji as the source of the control extract. In this experiment

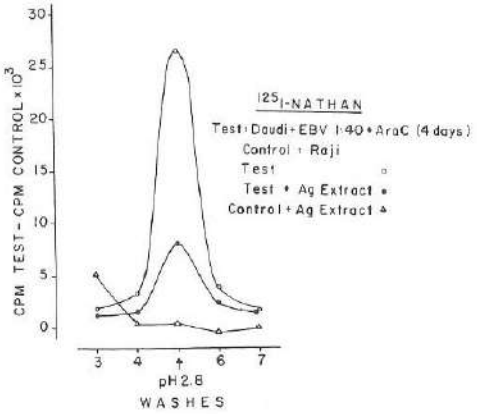

FIG. 9. Early antigen competition. Antigen extract prepared from Daudi cells superinfected with EBV (1:20) in the presence of AraC (20 μg/ml) 4 days previously. Test cells were the same. Labeled antibody used was Nathan (MA−, EA+, VCA+).

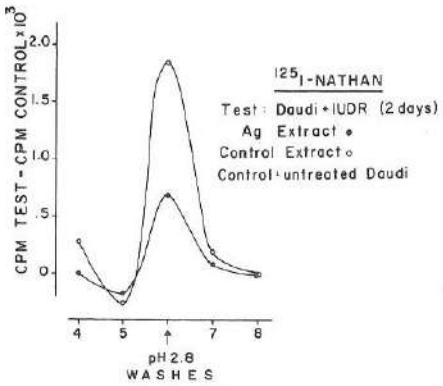

FIG. 10. Specificity of the EA competition: Comparison of the extracts from IUDR-treated Daudi cells with the extract from the same number of 698 cells in reducing the labeled Nathan antibody binding to the IUDR-treated Daudi targets. Control target cells were untreated Daudi.

the antigen extract decreased the counts per minute in the acid elution peak from the IUDR-induced Raji targets by 71% ($P < 0.005$) compared to the cells receiving control extract. The latter test confirms the specificity of this EA competition, since the only differences in the extracts used (both from Raji cells) was that one had been induced with IUDR which has been demonstrated to induce only EA (38).

DISCUSSION

Using ^{125}I-labeled human antibodies with specificity for EBV-determined antigens and human lymphoblastoid cell culture lines, the quantitative parameters of radioiodine-labeled antibody elution (RIE) have been further defined and antigen in a suspended form has been used to compete with the target cells for the antigen-combining sites of the labeled antibodies.

Abwao IgG which has specificity for all the EBV-determined antigens was used to quantitate antigens produced after superinfection of the Raji and Daudi cell lines. This method was found to be quite sensitive with detectable antigen differences between control and superinfected cells at 1:300 to 1:1000 dilutions of virus. Increases in the elution peaks were observed as the virus concentration increased. Adsorbed virus was not responsible for this binding of elutable antibody. The specificity of the reaction was confirmed since the control and test cells were identical except for the addition of EBV to the test cells 2–3 days prior to testing. EA immunofluorescence (IF), used as a quantitation of the number of infectious units in a culture after superinfection by EBV, has been demonstrated to be a very sensitive tool (39). Increments in the percentage of positive cells can be detected by IF in the same range of low virus concentration as the RIE test. The two tests are quantitating different parameters, however, and are thus complementary. The number of infectious units detected by EA IF is a reflection of the number of cells at a given virus concentration which are successfully infected. This is

influenced by the number of infectious particles in the virus preparation, the virus receptors on the cells, and the relative susceptibility of the cells to enter a productive cycle after superinfection. In the cell lines studied, this productive cycle is an abortive one (1, 16) and no new infectious virus is produced by the superinfected cells (16). Thus, the EA IF-positive cells counted represent the number of infectious virus particles present in the original superinfecting EBV preparation when a superinfection-sensitive cell line is used. The RIE, of course, gives no indication of the number of infectious units in a culture, but reflects a relative quantity of antigen expression in the entire population as well as the accessibility of the antigen to the labeled antibodies under these testing conditions.

The reasons for quantitating the amount of elutable antibody binding to the cells rather than the amount of antibody bound directly to the pellet deserves some attention. There are basically two justifications for this approach; first, it avoids, considerably, the high background of nonspecific binding to the sides of the tubes and cells, secondly, it allows one to evaluate the entire profile of counts in the washings and eluate rather than just two points, i.e., the test and control pellet. The latter of these two is by far the most important raison d'être for the RIE. If appropriate precautions are taken, the amount of nonspecific binding can be kept to a minimum in the radioiodine-labeled antibody binding (RIB) test (counting the pellet). Being able to evaluate the entire elution profile can eliminate many sources of error, however, which are inherent in counting the washed pellet only, regardless of precautions. Differences, for example, in the amount of antibody placed on the cells or slight differences in washing can be sources of error in the RIB. However, with the RIE one evaluates the counts in the washes also. Thus, if the test washings have higher counts than the control and there are no differences in specific antibody binding, there would be no elution peak from the test cells even though the counts are higher (see Fig. 3, 1:3000 virus dilution). This sort of evaluation becomes increasingly important as the differences in the test and control become smaller.

Using producer cells and control cells in parallel, increasing the antibody concentrations on constant cell numbers was shown to produce a linear increase in elutable antibody binding specifically to the test cells. Likewise, increasing the numbers of cells in the presence of a constant antibody concentration produced progressive increases in the amount of specific elutable-antibody binding.

It was previously demonstrated that the RIE detected the intracellular antigens EA and VCA as well as MA (27). It is likely that antibody enters the cells which are producing EA or VCA and binds specifically. Preliminary results with radioautography in this laboratory support this view (I. Ernberg, unpublished data). Furthermore, direct immunofluorescence for EA and VCA on living cells followed by fixation and counterstaining with Evans blue, gives the same percentage of EA- and VCA-stained cells as preparations which are fixed before exposure to the fluoresceine-conjugated antibodies (Hilgers, F., Rosén, A., Lamon, E. W., and Klein, G., unpublished data).

Antigen extracts in suspended form competed successfully with the test cells for the antigen-combining sites of the labeled antibodies, decreasing the peaks of elutable antibody binding to the test target cells. The competition reaction was specific. Extracts from frozen and thawed EBV determined antigen-possessing cells reduced the elutable antibody binding to the test cells compared to extracts prepared in an identical manner from the same number of control cells. The competition was sensitive and relatively quantitative. Decreasing concentrations of specific antigen extracts produced sequentially less competition and the extract from as few as 10^6 cells produced a reduction in the counts per minute of the antibody elution peak of more than one half. Since the titration was carried to only three dilution steps, the sensitivity or stoichiometry of this test for the detection of suspended antigen cannot, at the present time, be stated. These experiments have, however, illustrated the feasibility of such an approach. Reduction in the counts per minute may require antigen excess relative to the amount of specific labeled antibody present. As the quantity of specific antigen becomes smaller, in the range of antigen–antibody equivalence, the elution peak should be 50% of the positive control. The most sensitive antigen assays should be performed at a very low dilution of labeled antibody (i.e., in antigen excess relative to the target cells) so that small amounts of competing antigen would reduce the peak. The influence of antigen–antibody complex receptors on these cells on the results of RIE assays for antigen has not been determined.

The addition of antigen extract to control cells produced no significant amount of elutable antibody binding. This would seem to indicate that adsorbed antigen is not responsible for the binding of specific elutable antibody in this test. However, that adsorbed antigen in another physical state or at different concentrations than we have used could produce such a binding cannot be entirely excluded. Also, under these testing conditions, antigen antibody complexes are not binding and being eluted from the cells in detectable quantities. This could be due to a number of factors including the presence or absence of receptors for complexes on these cells (40) and the concentration of the antigen in relation to the antibody.

By selectively manipulating the Daudi cells to possess only MA and EA (EBV superinfection in the presence of Ara C) or only EA (IUDR induction) and by choosing an antibody which has specificity for EA and VCA but not MA (Nathan), we were able to demonstrate specific competition with suspended EA. Selective competition with other EBV-determined antigens using this method is possible. EA was chosen for these experiments because it is easily detected as a single entity by RIE.

For isolation and purification of antigens determined by EBV, a sensitive, specific, quantitative assay is needed. The RIE method appears to meet these requirements. We have demonstrated the feasibility of such an approach by using suspended antigen preparations to compete with the target cells for the antigen-combining sites of the labeled antibodies which have specificity for EBV-determined antigens. By selectively manipulating the cell lines to possess the desired antigens both as targets and as sources of soluble or sus-

pended antigen and proper selection of the antibody so that only one antigen is quantitated, as we have done with EA in the present report, the approach to isolation and purification of these antigens should be relatively straightforward. Recent work in this laboratory indicates that the EBV nuclear antigen (EBNA) can be detected in isolated nuclei from nonproducer lines by RIE, but not in nuclei from cell lines which do not possess the EBV genome and not in the intact nonproducer cells (41).

The isolation and characterization of these antigens is a requisite for an understanding of the virus–cell relationship in terms of the functions of these viral products on the molecular level.

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CHAPTER 3

Detection of Epstein-Barr virus-determined intracellular antigens in unfixed cells with labeled antibodies.

Detection of Epstein-Barr Virus-Determined Intracellular Antigens in Unfixed Cells With Labeled Antibodies[1,2]

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SUMMARY—Antigens determined by Epstein-Barr virus (EBV) were detected in human lymphoblastoid cell lines by radioiodine-labeled antibody elution (RIE) and immunofluorescence. Previous RIE studies with the use of suspensions of unfixed cells demonstrated that not only membrane antigen, but also intracellular viral capsid antigen (VCA) and early antigen (EA), were detected. In the present study, both restricted and diffuse subtypes of EA were detected by RIE with the use of cells that had been induced by 5-iodo-2′-deoxyuridine to produce EA. To see where the specific antibody was binding in these unfixed cell suspensions, fluorescein-labeled antibodies specific for EBV-determined antigens were used. The labeled antibodies entered and bound specifically to the intracellular antigens VCA and EA. When placed on the unfixed cell suspensions, these antibodies could be directly visualized within the cells after fixation and counterstaining with Evans' blue. The percentage of cells staining specifically for VCA or EA when the labeled antibodies were placed on the cells before fixation was the same as the percentage stained when the antibodies were added to parallel smears after fixation. We concluded that antibodies entered unfixed cells and bound specifically to EBV-determined intracellular antigens.—J Natl Cancer Inst 53: 949–955, 1974.

EPSTEIN-BARR VIRUS (EBV)-determined antigens, expressed in human lymphoblastoid cell lines, have been detected with various immunologic techniques (*1*). Immunofluorescence studies have localized EBV-associated antigens on the cell membrane as well as within the cell. The producer cell lines possess the membrane antigen (MA) (*2*), demonstrable on a high percentage of the cells by direct or indirect immunofluorescence of unfixed cell suspensions. A small percentage of the cells in a producer line possess intracellular viral capsid antigen (VCA) (*3*) and intracellular early antigen (EA) (*4*), both detectable by immunofluorescence on fixed cells. The EA, so named because it appears as an early viral product after superinfection by EBV of a nonproducer cell line (*4*), has been subdivided into restricted (R) and diffuse (D) subtypes which are antigenically distinct (*5*). MA, VCA, and EA are not present in the nonproducer cell lines under ordinary culture conditions. However, all the cell lines which possess the EBV genome, as detected by DNA/DNA (*6*) and complementary RNA/DNA (*7, 8*) hybridization studies, including the nonproducer lines, express the EBV nuclear antigen (EBNA) in all or most cells (*9*). EBNA is apparently present in much smaller quantities than the other antigens, since it cannot be detected by ordinary direct immunofluorescence. It becomes brilliantly apparent, however, with the use of anticomplement immunofluorescence. Some of the nonproducer cell lines can be induced to express EA by exposure to 5-iodo-2′-deoxyuridine (IUDR) (*10*).

The antibodies used to detect and define these antigens came from the sera of patients with infectious mononucleosis (*11*), Burkitt's lymphoma (*12, 13*), and nasopharyngeal carcinoma (*14–16*).

Some sera have antibodies specific for MA, VCA, and EA, whereas others lack reactivity for either MA or EA. A relatively small group of "discordant" sera react with EA and VCA but not MA (*13, 17*). Sera having reactivity with MA and VCA but not EA are common. Manipulations of the nonproducer cell lines by superinfection in the presence of cytosine arabinoside (Ara-C) induction, as well as use of producer lines, and selection of the proper reagent antibodies allowed us to detect and define the antigens determined by EBV as single entities.

It has previously been assumed that the binding of ^{125}I-labeled antibodies specific for the EBV-determined antigens to producer target cells was due entirely to MA (*18, 19*). However, it has recently been demonstrated with a sensitive radioiodine-labeled antibody elution (RIE) test that labeled antibodies specific for EBV-determined antigens were also detecting EA and VCA on unfixed cell suspensions (*20–23*). These experiments, however, gave no information regarding the location of the antigens to which the antibody bound. Either EA and VCA could be leaking to the cell surface, or the antibodies could be entering the cells and binding specifically. In the present report, we demonstrate that EA and VCA antibodies enter unfixed cells and bind specifically to these respective antigens.

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MATERIALS AND METHODS

Tissue culture lines.—The following lymphoblastoid cell lines were used. *a*) Raji, derived in 1963 from a Nigerian patient with Burkitt's lymphoma (*24, 25*), is a nonproducer cell line. *b*) Daudi, derived in 1967 from a Kenyan patient with Burkitt's lymphoma (*26*), has a low production of MA, VCA, and EA. Both Raji and Daudi express high amounts of MA, and EA and a small amount of VCA after superinfection with EBV (*9*). The expression of VCA can be completely suppressed by simultaneous treatment of the cells with Ara-C (*27*). Furthermore, the resident viral genome of these cells can be induced to express EA after exposure to IUDR (*9*). *c*) P3HR-1 cell line, derived in 1967 from a Nigerian patient with Burkitt's lymphoma (*28*), is a producer line that expresses MA, VCA, and EA. This line produces the EBV used for superinfection of the Raji and Daudi lines.

The cell lines were maintained as stationary suspension cultures with RPMI-1640 medium, supplemented with 15% fetal calf serum (FCS), 100 U penicillin/ml, and 50 μg streptomycin/ml, and were incubated at 37° C in a 5% CO_2 atmosphere. The cells were fed every 3 days and tested just before feeding.

EBV superinfection.—EBV was prepared from the medium of P3HR-1 cells by the polyethylene glycol concentration method (*29*) and stored at −70° C before use. One ml of the preparations induced 10–20% EA in 10^6 Raji cells at a dilution of 1:20. Cells, 10^6/ml, were incubated with EBV diluted 1:20 for 60 minutes at 37° C. Subsequently, the cell concentration was adjusted to 2.5×10^5/ml and 20 μg Ara-C/ml was added to inhibit VCA synthesis. The cells were tested 4 days after being superinfected parallelly with untreated controls.

EA induction.—IUDR, 25 μg/ml, was added to Daudi or Raji cells in fresh medium. The cells were harvested 3 days later parallelly with untreated controls.

Sera.—The following sera were used: *a*) Abwao contains antibodies to MA, VCA, and EA; *b*) Mutua contains antibodies to MA and VCA but not to EA; *c*) Nathan reacts with VCA and the D component of EA but not with MA; *d*) Martha is specific for MA, VCA, and the R component of EA; *e*) Patrice reacts with VCA and the D component of EA; and *f*) Odour reacts with MA, VCA, and the R component of EA. In these experiments, sera *c–f* were used only on cells that had been induced to express EA by exposure to IUDR. Thus the only pertinent specificity in this study is for the EA complex.

Radioiodine antibody labeling.—IgG, 200 μg, at a concentration of 2 mg/ml in 0.15M sodium phosphate buffer, pH 7.4, was labeled with 1 mCi ^{125}I by the chloramine-T (*30*) method as described in (*18, 19*). Then the IgG was placed on a Dowex (AG1X-2, 200–400 mesh) column prepared in a Pasteur pipette prewashed with 5% FCS in phosphate buffer. The labeled IgG was eluted from the column with 2 ml of the 5% FCS, which resulted in an eluted volume of 2.6 ml. The preparation was stored at 4° C in lead shielding before use.

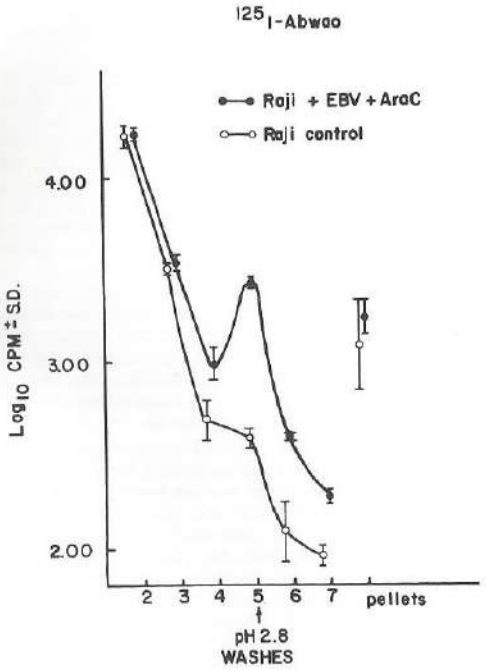
RIE.—In triplicate tubes, prewashed with FCS, 10^6 test or control cells were added. Then 50 μl of a ^{125}I-labeled IgG was added to each tube and incubated for 1 hour at 4° C. The cells were washed 4 times with cold medium containing 10% FCS, after which the specifically bound antibody was eluted with 1 ml 0.1M glycine HCl, pH 2.8, by incubation for 30 minutes at 37° C. Subsequently the cells were washed 2 more times with medium. All washings and the eluate were collected separately and their ^{125}I radioactivity was determined in a Nukab gamma counter. The $\log_{10}$ mean and standard deviation of the counts per minute (cpm) of the triplicate supernatants were calculated. Also the absolute difference in cpm between the test and control was calculated to show the amount of elutable antibody binding specifically to the test cells.

Immunofluorescence.—Unfixed suspensions containing $2–4\times10^5$ cells/tube were incubated for 45 minutes at 4° C with fluorescein isothiocyanate (FITC)-conjugated IgG (specificities and dilutions indicated in "Results"). The cells were washed 3 times with balanced salt solution (BSS) and placed on a glass slide. The smears were then fixed in acetone for 10 minutes at room temperature and air-dried. Counterstaining was done with 0.01% Evans' blue in distilled water for 10 minutes. The slides were washed in distilled water, air dried, and mounted in 50% glycerol in BSS. Parallel cell preparations were exposed to the FITC-conjugated IgG *after* acetone fixation of the smears (the usual method for detection of intracellular antigens). The smears were incubated with the conjugates for 30 minutes at 37° C in a moist chamber, after which they were washed twice with BSS and once with distilled water, counterstained with Evans' blue, and then mounted in 50% glycerol. For detection of EA, IUDR-induced Daudi cells were used as targets. (No VCA is produced in IUDR-treated cells.) For the detection of VCA, P3HR-1 cells were used as targets and the MA+, VCA+, EA− Mutua conjugate was used to stain the antigens. Specificity was demonstrated by blocking with unlabeled Abwao serum (preincubation of the cell suspensions or smears with undiluted serum followed by application of the labeled antibody). The percentage of positive cells was determined with a fluorescence microscope equipped with a Ploem-Orthoplan illumination system, under an oil immersion ×100 objective.

RESULTS

RIE

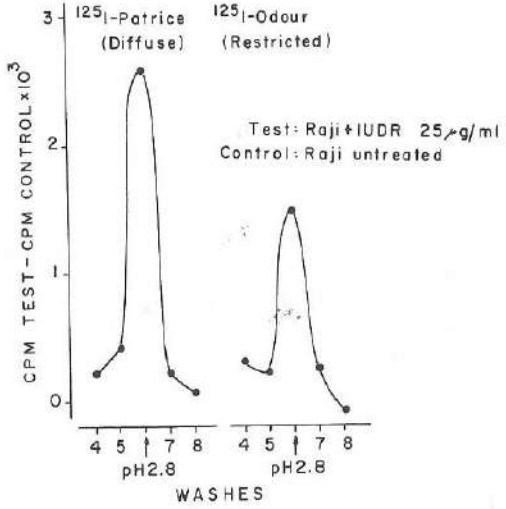
Text-figure 1 shows the RIE profile ($\log_{10}$ scale) with ^{125}I-labeled Abwao IgG tested on Raji cells superinfected with EBV in the presence of Ara-C as the test cells and untreated Raji control cells. The cpm in the test and control supernatant washings decreased parallelly until the specifically bound antibody was eluted with acid. The striking difference between the test and control supernatants in the



TEXT-FIGURE 1.—RIE profile of ^{125}I-labeled Abwao IgG (MA+VCA+EA+) diluted 1:16 (0.25 μg IgG/tube) on Raji cells superinfected with EBV in the presence of Ara-C (20 μg/ml) as the test cells and untreated Raji as control cells. *Horizontal axis* indicates sequential washes, elution, and pellet. *Vertical axis* indicates the log mean±SD of cpm of triplicate washings and elution.

low-pH eluate represented elutable antibody binding to EA and MA, since VCA was blocked with Ara-C. With the use of unlabeled sera specific for MA or EA to compete with the labeled antibody for MA or EA antigenic determinants as described in (*21*), over half the reactivity in this experiment (reflected by the cpm in the elution peak) was due to elutable antibody binding to the intracellular EA. In this experiment, there was no significant difference in the cpm remaining on the cell pellets of the test and control cells after the acid elution, which indicated that the specifically bound antibody was eluted from the cells by acid.

It was previously demonstrated that EA could be detected as a single entity by RIE if the cells were induced by exposure to IUDR (*20–23*). In the following experiment (text-fig. 2), IUDR-treated Raji cells, in parallel with untreated control cells, were tested by RIE with labeled IgG specific for the D (Patrice) and R (Odour) components of EA. Both D and R EA's were detectable by RIE. The confined localization of R EA in cytoplasmic inclusions would thus suggest that the antibodies entered the cells expressing this antigen.



TEXT-FIGURE 2.—Detection of D and R EA's by RIE. Test cells were Raji and were treated with IUDR, 25 μg/ml, for 2 days. Control cells were untreated Raji. The cpm of control cells were subtracted from cpm of test cells to show specific amount of elutable binding. Labeled IgG's were diluted 1:3 (1.3 μg IgG/tube).

Immunofluorescence

To see whether antibodies were entering these unfixed cells and binding specifically to intracellular antigens, experiments were done with FITC-labeled antibodies placed parallelly on the unfixed or fixed cells. After the unbound antibody was removed by being washed, the cell suspensions were then fixed on a slide with acetone, counterstained, and examined for intracellular fluorescence. Table 1 summarizes the results of these experiments. The EA expression of the IUDR-induced Daudi cells could be detected equally well by the FITC-labeled antibodies, whether the cells were exposed to the antibodies before or after fixation. This was true of the R component. The D component of EA, however, was detectable in a slightly higher percentage of the cells when they were stained after fixation. VCA was detectable in the P3HR-1 cells exposed to the antibodies before fixation in percentages equal to those in the preparation exposed to antibody after fixation. The specificity of the reaction was demonstrated by the ability of unlabeled Abwao serum to block the reaction in either situation. To illustrate the localization of the intracellular fluorescence, figure 1 shows photomicrographs of 3 antigenic specificities (VCA, R EA, and D EA) stained by the appropriate labeled antibodies being placed on the cells before and after fixation. The intracellular localization of the antibodies was striking in either situation. The importance of the Evans' blue counterstain should be pointed out: If smears were examined without counterstain, the amount of fluorescein taken up nonspecifically by the unfixed cells made the smear impossible to read.

TABLE 1.—*EA and VCA fluorescence on fixed and unfixed cell preparations, with the use of FITC-conjugated sera and counterstain with Evans' blue*

Target cells	Conjugate	Dilution	Pertinent specificity	Blocking serum	Antibodies placed on cells: Before fixation (% positive cells)	Antibodies placed on cells: After fixation (% positive cells)
Daudi+IUDR	F-1 Martha	1:20	R EA	—	3. 3	3. 4
Daudi+IUDR	F-6 Nathan	1:20	D EA	—	3. 4	5. 2
P3HR-1	F-55 Mutua	1:20	VCA	—	3. 7	3. 2
P3HR-1	F-55 Mutua	1:20	VCA	Abwao	0. 5	0. 5

Evans' blue, however, almost completely suppressed the nonspecific staining, but had no detectable effect on specific EA and VCA staining.

DISCUSSION

Radioiodine-labeled antibodies have been used to detect MA's in various systems, including histocompatibility (H-2) antigens of the mouse (*31*), tumor-associated antigens on Friend virus-induced lyphoma cells and 3-methylcholanthrene-induced sarcoma cells (*32*), and polyoma-induced (*33*) and simian virus 40-induced surface antigens (*34*).

It has been assumed that only MA binding was quantitated by radioiodine-labeled antibodies specific for EBV-associated antigens on human lymphoblastoid cell lines (*18, 19, 35*). Recently, however, with unfixed lymphoblastoid cell lines as targets in an RIE test, not only EBV-determined MA, but also the intracellular VCA and EA, were detected with labeled antibody (*20–23*). In the present study, we demonstrated the mechanism by which this detection of the intracellular antigens occurs. With FITC-labeled IgG from sera with high reactivity for VCA and EA, we demonstrated that most VCA- or EA-positive cells were stained in unfixed cell preparations. This indicated that labeled antibodies could detect intracellular antigens by entering the VCA- or EA-positive cells and binding specifically to these respective antigens rather than the leaking of the antigens to the cell surface. Conceivably, the sensitive RIE test could be quantitating some VCA and EA at the cell surface; however, the present study demonstrated that the intracellular VCA and EA are available to the specific antibodies that enter these cells. Thus any EA or VCA binding at the surface would be small compared to the antigen binding within the cells, especially since the quantity of EA and VCA is so much greater within the cell. The mechanism of entrance of the antibody into cells expressing VCA or EA is probably not due to an active uptake or endocytosis, since the tests were done at 4° C. It would seem likely that the entrance of the antibodies into the cells and subsequent specific binding to intracellular antigens are due to passive entry through damaged membranes. Recently Mizuna and Osato (*36*) demonstrated that most VCA- and EA-positive cells were membrane damaged, as assessed by trypan blue uptake. However, they also found a minority of VCA- and EA-positive cells that did not take up the dye. This could account for the slightly higher number of D EA-positive cells observed when the cells were exposed to the antibodies after fixation.

Cells committed to a productive cycle, signaled by the expression of EA, have a rapid decrease in host cell macromolecular synthesis and a concomitant increase in synthesis of viral components (*37*), i.e., viral DNA replication and finally VCA production. The endpoint of the productive cycle is cell death and production of complete virus particles. Since cytopathic effects are associated with the expression of EA and VCA, it would be pertinent to ask whether the RIE was simply reflecting this cytopathic effect by a greater trapping of IgG molecules nonspecifically in the test cells than the control cells. Overwhelming evidence indicates that this is not so. First of all, labeled IgG from EBV-negative human serum (NHS) does not bind to the cells (*21*). Also, unlabeled serum with the appropriate EBV specificity can compete with the labeled EBV-reactive IgG for intracellular antigenic sites, whereas there is no such competition by NHS. Finally, we (unpublished observations) demonstrated in repeated experiments that high percentages of dead cells per se do not increase the amount of elutable antibody binding (*20*). However, we probably are not quantitating all the intracellular VCA and EA by RIE. It has been demonstrated that more intracellular antigens are available for antibody binding in cells disrupted by freezing and thawing than intact "viable" cells (Ernberg I, Klein G: Unpublished observations). That the RIE can be used for a relative quantitation has been documented (*20–23, 38*). However, the intracellular antigens detected by this method probably involve only those cells that are late enough in the productive cycle to have membrane damage sufficient to allow entry of the IgG.

Recently Boone et al. (*35*) attempted to isolate EBV-determined MA from fractionated producer cells treated with MA, VCA-reactive labeled antibody, and normal globulin from NHS in a paired radioiodine-labeled antibody test before disruption of the cells. Subsequent fractionation of the cells showed specific antibody binding to only the heavy fractions containing naked virus particles. It was concluded that MA-positive cells in producer cultures were possibly carrying EBV- and MA-positive debris

adsorbed from the perinuclear and endoplasmic membranes of degenerating infected cells. However, after incubation of the producer cells with their paired labeled antibodies, the cells were washed 6 times with large volumes of cold saline before disruption and fractionation. Under these conditions one would not expect to have any MA antibody left attached to the cells. Our studies showed that very little antibody corresponding to MA reactivity remained, even after 5 washes with small volumes of cold medium containing calf serum (*21*). High quantities of VCA and EA were detectable, however, after 5 washes. Thus, the MA- and the VCA-reactive antibody used in the fractionation studies probably quantitated only VCA because the washing procedure eliminated MA-reactive antibody.

Furthermore, we recently showed that MA and EA, as detected by RIE, reflected active antigen synthesis after superinfection of a nonproducer line with EBV (*22*). MA and EA expression could be suppressed by the addition of the protein synthesis inhibitor, puromycin. The antigens detected were not due to adsorbed virus, since performing the RIE 1 hour after the addition of virus gave negative results (*23*). The earliest detection of MA or EA was 2 days after superinfection (*22*). Thereafter, on days 3 and 4, the amount of EA detectable by RIE increased significantly, whereas MA increased only slightly on day 4 after superinfection (*22*). The increase in EA detection probably reflects both increased antigen synthesis and greater access for the antibodies via membrane damage.

Most experiments with radioiodine-labeled antibodies to detect cellular antigens on unfixed cell populations are interpreted based on the assumption that the antibodies are binding to only cell-surface antigens. Our present data indicate that this assumption is not always valid, particularly with respect to virally determined antigens when some of the cells are undergoing cytopathic changes related to viral antigen production in the course of a productive cycle.

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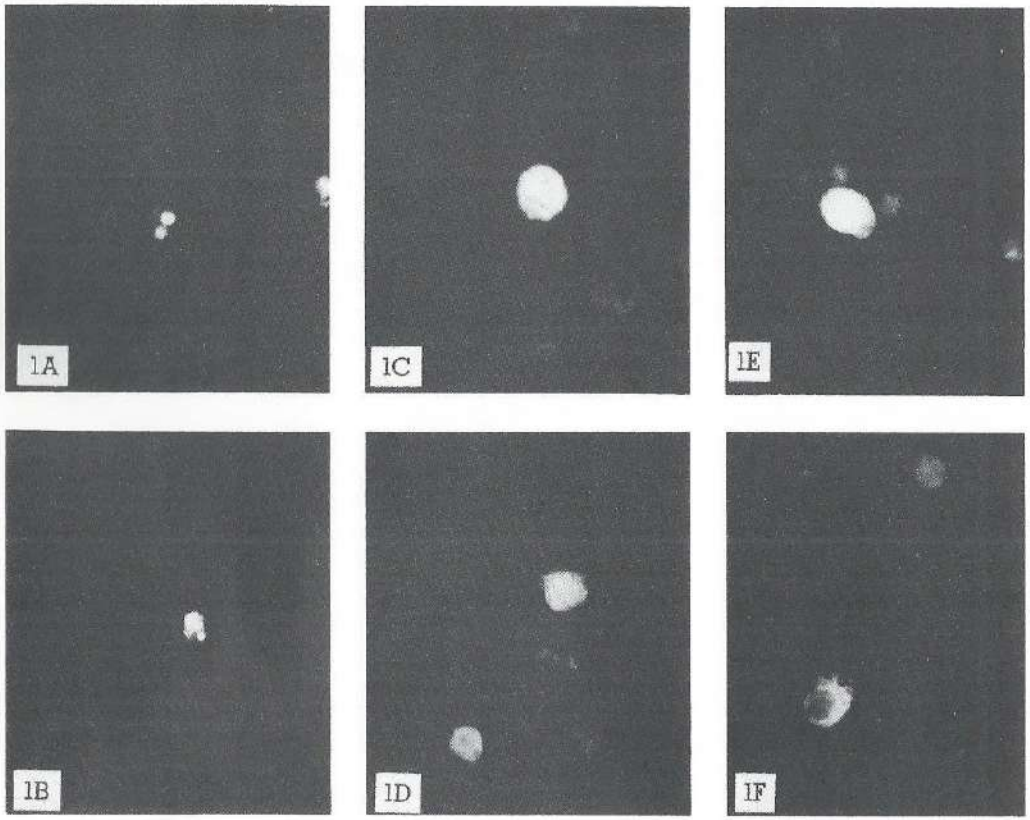

FIGURE 1.—Intracellular localization of FITC-labeled antibodies reacting with EBV-associated antigens. A, B: Target cells were Daudi and were treated with IUDR for 3 days. Fluorescein-labeled IgG (Martha, R EA specificity) was placed on cells before fixation in A or after fixation in B. C, D: Target cells were same as A and B. FITC-labeled Nathan IgG (D EA specificity) was placed on the cells before fixation in C or after fixation in D. E, F: Target cells were P3HR-1. FITC-labeled Mutua IgG (VCA specificity) was placed on cells before fixation in E and after fixation in F.

CHAPTER 4

An immunofluorescence technique with counterstain on fixed cells for the detection of antibodies to human herpesviruses; antibody patterns in patients with Hodgkin's disease and nasopharyngeal carcinoma.

An Immunofluorescence Technique with Counterstain on Fixed Cells for the Detection of Antibodies to Human Herpesviruses; Antibody Patterns in Patients with Hodgkin's Disease and Nasopharyngeal Carcinoma*

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KEYWORDS

- Hodgkin's disease
- Nasopharyngeal carcinoma
- Herpesviruses
- Immunofluorescence
- Sero-epidemiology

LIST OF ABBREVIATIONS

ACIF	anti-complement immunofluorescence
ACIFA	anti-complement immunofluorescence absorption
ANF	anti-nuclear factor
BL	Burkitt's lymphoma
CF	complement fixation
CLL	chronic lymphocytic leukemia
CMV	cytomegalovirus
CPE	cytopathic effect
CS	calf serum
EBSS	Earle's balanced salt solution
EBV	Epstein-Barr virus
EA	EBV-determined early antigen
EBNA	EBV-determined nuclear antigen
MA	EBV-determined membrane antigen
VCA	EBV-determined viral capsid antigen
FCS	fetal calf serum
GMT	geometrical mean titer
HD	Hodgkin's disease
HEDLF	human embryonic diploid lung fibroblasts
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
ID	immunodiffusion
IF	indirect immunofluorescence
IM	infectious mononucleosis
MC	mixed cellularity HD
NPC	nasopharyngeal carcinoma
NS	nodular sclerosing HD
PBS	phosphate buffered saline
PFU	plaque forming unit
RK 13	rabbit kidney cell line
Vero	African green monkey kidney cell line
VZV	varicella-zoster virus

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SUMMARY

An indirect immunofluorescence (IF) test on fixed cells with Evans' blue counterstain is described for all four herpesviruses, *i.e.* herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2), varicella-zoster virus (VZV), cytomegalovirus (CMV) and Epstein-Barr virus (EBV). Comparison with immunodiffusion (ID) for HSV-2 and with ID and complement fixation (CF) for VZV and CMV demonstrated the specificity and high sensitivity of the IF test. Also introduced is a modification of the anti-complement immunofluorescence (ACIF) test for EBV-determined nuclear antigen (EBNA), permitting simultaneous titration of antibodies to this nuclear antigen and of the anti-nuclear factor (ANF). IF titers to the various viruses in our test series are very much higher to EBV (1:964) than to CMV (1:166), HSV (1:107) and VZV (1:59).

Sero-epidemiology studies to these viruses in patients with Hodgkin's disease (HD) in the Netherlands revealed the following pattern, (1) in the nodular sclerosing (NS) HD there is a 4x (significant) elevation in antibody titer to EBV-VCA, but no elevation to EBV-EA and EBNA and (2) in the mixed cellularity (MC) HD a 10x (significant) elevation to both EBV-VCA and EA, but no elevation to EBNA is found compared to the control groups. These patterns in NS and MC HD are different from the pattern in nasopharyngeal carcinoma (NPC) with elevations in antibody titers to EBV-VCA and EA as well as to EBNA. Antibody titers to HSV, VZV and CMV are not significantly elevated in both HD and NPC.

INTRODUCTION

Since the discovery of the Epstein-Barr virus (EBV) in tissue culture cells derived from a Burkitt's lymphoma (BL) biopsy (1), this herpesvirus has

been identified as the causal factor of infectious mononucleosis (IM) (2). It is also thought to be associated with a number of neoplastic diseases, such as BL itself and nasopharyngeal carcinoma (NPC) (3, 4, 5, 6, 7 and for review 8), Hodgkin's disease (HD) (9, 10), chronic lymphocytic leukemia (CLL) (11) and non-neoplastic diseases such as sarcoidosis (12, 13), systemic lupus erythematodes (14) and lepromatous leprosy (15). Following the discovery of EBV by electron microscopy (1) and the use of indirect immunofluorescence (IF) by Henle and Henle, 1966 (16) for detection of the virus capsid antigen (VCA) in lymphoblastoid cell lines, derived from BL tumor biopsies, Klein *et al.*, 1966 (17) were able to demonstrate an EBV-determined membrane antigen (MA), present on all tumor biopsy cells. Reedman and Klein, 1973 (18) obtained strong evidence by an anti-complement immunofluorescence (ACIF) test for the presence of an EBV-related nuclear antigen (EBNA) in African BL with two exceptions (19). Recently Reedman *et al.*, 1975 (20) confirmed this observation with the highly specific anti-complement immunofluorescence absorption (ACIFA) test. In fact it has previously been shown (21), that BL biopsies contain EBV-specific DNA.

A similar story can be told for NPC. The epithelial-derived cells of this anaplastic carcinoma also contain EBNA- and EBV-specific DNA (22, 23). In the other neoplastic diseases mentioned above, *i.e.* HD and CLL, associated with EBV, no EBV-specific antigens or DNA have been found in the tumor cells (19, 23). However, in HD the tumor cell has not been recognized as yet and is thought to represent a minor population (24) amongst reactive lymphoreticular cells.

In fact, the association of these two diseases with EBV is based on sero-epidemiologic findings only. Thus, elevated antibody titers to EBV-VCA and EA have been found in both treated and untreated patients suffering from these diseases (9, 10, 11, 25, 26, 27). Although it has been speculated initially that EBV was the causitive factor for HD and CLL, it is generally accepted now that the elevation in antibody titers reflects an imbalance in the immune status. The latter hypothesis is supported by the findings of Henderson, 1973 (26) of elevated antibody titers to EBV as well as varicella-zoster virus (VZV) in HD.

While the association of EBV with human neoplasia, especially BL and NPC, is a strong one, the association between the other three herpesviruses, *i.e.* herpes simplex virus type 1 and 2 (HSV-1 and HSV-2), VZV, and Cytomegalovirus (CMV) and human neoplasia is weak. For example, HSV-2 has been claimed to be the possible causal factor for cervical carcinoma, mainly on the basis of sero-epidemiology (28, 29, 30), while the presence of specific nucleic acids in the tumors is disputed (31, 23). Nevertheless, evidence is increasing that HSV-1, HSV-2 and CMV are carcinogenic in experimental animals under certain conditions (32, 33).

Strangely enough, sero-epidemiologic studies for human herpesviruses have been carried out with IF techniques mainly for EBV and the less sensitive complement fixation (CF) technique for HSV, VZV, and CMV. This has led to a misconception that in BL only antibody titers to EBV, but not to the other herpesviruses were elevated. However, utilizing IF techniques for all four herpesviruses (34) significant elevations in antibody titers to EBV, VZV, and CMV were demonstrated in BL patients. This was one of the reasons to further investigate the sero-epidemiology of all four herpesviruses in HD with the in specificity and sensitivity comparable IF technique.

Another reason is the interesting difference between the epidemiology of HD in the Netherlands and most other countries (35), since the incidence of HD in the young age group (20-30 years of age) in the Netherlands is comparitively high.

MATERIALS AND METHODS

Cells

Lymphoblastoid cell lines used were: P3HR-1, Jijoye, Daudi and Raji, kindly provided by Dr. G. Klein, Karolinska Institute, Stockholm, Sweden. Cells were maintained as stationary suspension cultures in RPMI 1640 tissue culture medium supplemented with 20% fetal calf serum (FCS). Medium was changed twice a week, reducing cell counts to approximately 3×10^5 cells per ml. In order to obtain a sufficient number of VCA positive cells, P3HR-1 or Jijoye cells were adjusted to 1×10^6 cells per ml and kept for 4 or 5 days in arginine-depleted medium with 5% FCS at 34°C (36). EBV-EA was induced by superinfecting Daudi cells with EBV and treatment with cytosine arabinoside (37). For EBNA titrations we used either untreated Raji or Daudi cells, the latter with a low background of less than 1% of VCA and EA positive cells.

With HSV-1 or HSV-2 the RK 13 cell line was infected and for VZV or CMV human embryonic diploid lung fibroblasts (HEDLF) were used. The RK 13 cells, kept as monolayer culture, were trypsinized once a week with 2% trypsine-versene and grown in Eagle's Minimal Essential Medium (MEM) supplemented with 10% calf serum (CS). Cells were fed two times a week with 2.5% CS containing MEM. HEDLF, also kept as monolayer culture, were trypsinized twice a week and started in MEM with 10% CS. Both RK 13 and HEDLF were grown in Flow tissue culture flasks no. 50 (Flow Laboratories Ltd., Scotland). After virus infection, cells were fed with MEM containing 2.5% CS.

Viruses

EBV, used for superinfection of Daudi cells, was obtained from the P3HR-1 cell line and concentrated by the polyethylene-glycol method of

Adams, 1973 (38). It was kindly provided by Dr. A. Adams (Karolinska Institute, Stockholm, Sweden).

HSV-1 and HSV-2 were isolated from an oral blister and a vaginal smear respectively. Typing was based on pock size on the chorioallantois membrane of a chicken embryo and on the colony form and giant cell formation on RK 13 and Vero cells (for review see Nahmias and Dowdle, 1968 (39)).

The VZV isolate 69-115 from a zoster patient and the CMV "Reints" isolate were used in all experiments. These viruses were kindly provided by Dr. K.W. Slaterus, Wilhelmina Gasthuis, Amsterdam, who did the typing on the basis of colony form and CF results with known sera. All viruses were kept at −70° C.

Infection procedures

Superinfection with EBV for EA induction. 60×10^6 Daudi cells were pelleted, resuspended in 10 ml of a 1:40 diluted virus preparation and incubated 1 hour at 37°C. The cells were than resuspended in 200 ml RPMI 1640 tissue culture medium containing 15% FCS and 20 μg/ml cytosine arabinoside (to inhibit DNA synthesis and thus prevent VCA production). Cells were harvested after 2 or 3 days.

Infection with HSV-1 and HSV-2. One seeding stock of both viruses was used in all tests. HSV-1 contained 3×10^7 plaque forming units (PFU) per ml and HSV-2 2×10^6 PFUs per ml. After isolation both stocks were passaged twice in Vero cells and once in RK 13 cells. Infection was carried out before the cells became confluent with 1.5 ml of a 10^{-3} dilution of HSV-1 and with 1.5 ml of a 10^{-2} dilution of HSV-2. Cells were than incubated for 30 minutes at room temperature. Medium was added and cells were further incubated at 37°C until sufficient cytopathic effect (CPE) (80-90%, usually after 2-3 days) was obtained. Cells were then harvested for IF by scraping with a "rubber policeman". These cells were resuspended with an equal number of uninfected cells.

Infection with VZV and CMV. Seeding stocks were maintained in MEM containing 10% DMSO. The procedure is the same as for HSV-1 and HSV-2. When infected cells showed 80-90% CPE they were harvested by trypsinization with 2% trypsine-versene and mixed with uninfected cells.

Indirect immunofluorescence (IF) and anti-complement immunofluorescence (ACIF).

The IF method used is essentially the same as described earlier for oncornaviruses (40). The ACIF test is according to the method of Reedman and Klein, 1973 (18) with some modifications.

Clean standard microscopic slides were covered with a hydrophobic teflon film (Hiflon, Hylin Works, Enfield, Middlesex, U.K.) with 8 distinctive free wells in it. Cells were washed three times in Earle's balanced salt solution (EBSS) and adjusted to a concentration of 1×10^6 per ml. One 50 μl drop was put on each well and permitted to dry overnight at room temperature. Cells were fixed in acetone for 10 minutes and stored at −20° C until use.

For ACIF the preparation of the slides was somewhat different. Cells were suspended in EBSS/distilled water (1:1) in a concentration of 10×10^6 per ml. To each spot 5 μl of this cell suspension was added and smeared with a Pasteur's pipet. After quick drying with a hairdryer, cells were fixed for 10 minutes in acetone and washed with distilled water. Dried slides were stored at −90° C. This procedure permits their use for several months.

Antigen positive cells were always mixed with antigen negative cells, *i.e.* HSV, VZV and CMV infected cells with non-infected cells and EBNA positive cells with GRSL cells (41) or YAC cells (42). These murine cells were used to enable detection of the ANF on the same slide. For EBV-VCA and EA this procedure is not needed because for these antigens no more than 5% and 20% positive cells respectively are present. A series of 50 μl drops of fourfold dilutions in saline (starting with undiluted serum) was placed on the spots. The last spot was kept for saline, *i.e.* the conjugate control. After 1 hour incubation at 37°C in a humified chamber, slides were washed twice in saline and twice in distilled water. Slides were then dried with a hairdryer. The extra step for ACIF required one drop of human complement, diluted 1:10, on each spot and incubation for 1 hour at 37°C in a humified chamber. Washing was done with extra care, twice with saline and twice with distilled water. One drop of conjugate (fluorescein conjugated anti-human immunoglobulin in IF or fluorescein conjugated anti-human complement (C3) in ACIF) was added to each spot and the slides were incubated for 1 hour in a humified chamber at 37°C. After washing, twice with saline and once with distilled water, cells were counterstained with Evans' blue (0.01% solution in distilled water) for 5 minutes. After washing twice with distilled water, cells were mounted in glycerol/PBS (1:1) and covered with a coverslip (20x50mm). Fluorescence was read under a Zeiss fluorescence microscope. Slides were stored at −20°C and could be re-examined up to several months after preparation without loss of activity.

Titer determination

Titers were determined as the highest dilution giving specific fluorescence. If there was only weak specific fluorescence left in a certain spot, the dilution corresponding with this spot was called the endpoint. If there was a sharp change from positive and clear fluorescence in one spot to complete absence of fluorescence in the next spot the titer was judged in between the two fourfold dilutions. Sera were always titrated without any

previous knowledge of the diagnosis to the investigator.

The reproducibility of the IF test was tested by repeating the serum dilutions several times. Furthermore, the different slides were read by different experienced investigators. The reproducibility appeared to be good, with no more difference than 1 twofold dilution step between the subsequent titrations and the different readers. In order to minimize a possible error however, all titers used in this and other papers were read by one investigator only. The counterstain Evans' blue is essential for accurate and rapid titer determinations, since the blueish-green autofluorescence of cells is changed into red. See also Hilgers *et al.*, 1974 (43) and Klein *et al.*, 1974 (44).

Immunodiffusion (ID)

A 50% cell extract in saline of approximately 100% infected cells (HSV-2, VZV or CMV) was prepared by freezing and thawing and successive sonication in an International Sonifier at 1.5 amps for 30 seconds. The extract was clarified by centrifugation at 3000 *g* for 30 minutes. The supernatant was used as a crude antigen preparation. For ID Hyland 2% agarose plates with well to well distance of 0.5 cm were used. The central well was filled with the antigen and the five surrounding wells with undiluted sera. Wells were filled four times in the course of an hour and plates were then placed in a humified chamber at room temperature. Reactions were scored after 24 and 48 hours.

Complement fixation (CF)

The CF tests were carried out by Dr. K.W. Slaterus (Wilhelmina Gasthuis, Amsterdam), by a macromethod using 4 amboceptor units, 5 50% complement units and 4 antigen units. The reactions were scored after overnight incubation at 4°C.

Sources of Sera

1 – Sera from Tunesian patients with NPC and from 20 age and sex matched healthy Tunesian controls were obtained from Dr. G. de Thé, IARC, Lyon. France.

2 – Sera from patients with HD and with a wide variety of neoplastic diseases, were collected in the clinic and out-patient department of the Netherlands Cancer Institute, Amsterdam, by Dr. F. Cleton.

3 – Sera from age and sex matched controls were collected from healthy blood donors by Dr. P.J. de Vries, Bacteriological Laboratory, GG & GD, Amsterdam.

4 – The paired sera from Figures 1 and 2 were obtained from Dr. K.W. Slaterus.

All sera were stored at –20°C.

Human complement for ACIF

Blood was taken from an EBV and ANF negative donor and permitted to clot at 4°C. After clotting, the serum was divided in 0.2 ml samples and stored at –90°C. In the ACIF test the complement was diluted to 1:10 in EBSS with Ca^{++} and Mg^{++}.

Conjugates

Swine anti-human immunoglobulin (IgG, IgM and IgA), swine anti-human IgG and swine anti-human IgM, all conjugated with FITC, were purchased from Nordic Diagnostics, Tilburg, The Netherlands.

Goat anti-human complement C3 (β_{1c}/β_{1a} globulin), conjugated with FITC, was obtained from Hyland Laboratories Inc., Cosa Mesa, California, 92626, USA.

All reagents were used at a 1:20 dilution in PBS.

Immunofluorescence on HSV-1 infected cells was hampered by the fact that conjugate controls for swine anti-human immunoglobulin, used in all tests, showed some non-specific reactions. HSV-2 infected cells did not show this. Since no differences in antibody titers to HSV-1 and HSV-2 were found, HSV-2 infected cells were used for the titration of antibodies to the HSV group specific antigens.

Fluorescence microscope

Immunofluorescence was read with a Zeiss fluorescence microscope. A 54x planapochromatic oil immersion objective was used in combination with 6.3x occulars. A Xenon lamp (Osram, 150 W) served as the light source, with heat and excitation filters numbers BG 38 and AL 470. Barrier filters used were K 510 in combination with a dichroic mirror TK 495.

Statistical analysis of the IF titers

For statistical analysis we used the Student's t-test. The level of significance chosen was $P \leqslant 0.01$.

RESULTS

In order to ascertain the specificity and sensitivity of the IF test for HSV, VZV, and CMV, we compared this test with CF and ID for these viruses. A total of 146 human sera were tested for antibodies to HSV-2 with the IF and ID tests. Table 1A shows that 126 sera (86%), 95 of which were positive and 31 negative, gave concordant results in both tests. None of the sera was positive in ID and negative in IF, but 20 sera were negative in ID and positive in IF. In these 20 sera IF titers were scored up and including 1:32. Table 1B shows 10 ID negative and 31 ID positive sera with

TABLE 1

COMPARISON OF IF TITERS AND ID RESULTS FOR HSV-2 IN 146 HUMAN SERA

A		POS. IF	NEG. IF
POS. ID		95	0
NEG. ID		20	31

B							
IF TITER		2	4	8	16	32	64 and >*
NEG. ID + POS. IF	(20)	1	3	6	7	3	0
POS. ID +	(95)	0	0	0	14	17	47**

In parenthesis serum number with this combination Pos. ID and Pos. IF.

* Reciprocal of serum dilution

** Total serum number with a certain IF titer

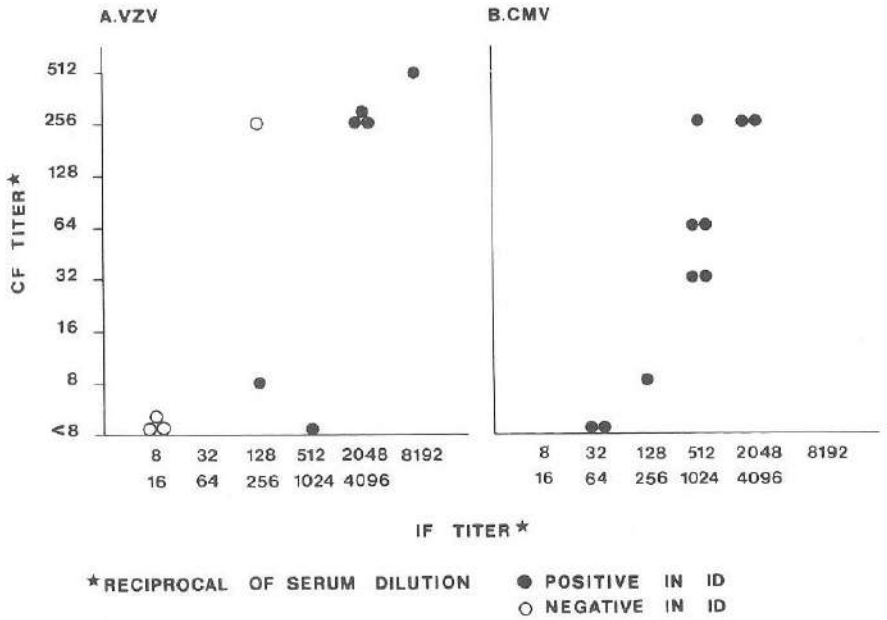

FIGURE 1. Comparison of complement fixation (CF) titers, immunofluorescence (IF) titers and immunodiffusion (ID) results for varicella-zoster virus (VZV) (A) and for cytomegalovirus (CMV) (B) in the first and second serum sample after onset of the disease.

IF titers of 1:16 and 1:32. These results indicate that the IF titers were scored with great reliability and furthermore that the IF test is roughly 10-20x more sensitive than ID under these circumstances.

The results of the comparison between IF, ID and CF tests for antibodies to VZV and CMV are shown in Figure 1. For both viruses we studied five paired sera with a fourfold increase in CF titers. By IF the same rise in titers was found. For CMV there was a good correlation between all three tests, *i.e.* CF titers of 1:8 and higher gave IF titers of 1:256 and higher and positive ID results. However, 2 sera were negative in CF and positive in IF and ID. In case of VZV positive sera with a low titer (1:8 and 1:16) by IF were negative both in CF and ID. High IF titers ($\geq$ 1:128) corresponded with positive reactions by CF and ID with 2 exceptions. One exception was comparable with the situation for CMV in case of the IF-ID positive, but CF negative serum. The other exception was IF-CF positive, but ID negative, which is less easy to interpret.

It is clear that the IF test for HSV, CMV, and VZV is a specific, sensitive and practical technique for performing sero-epidemiology. It permits a good comparison with the results obtained for these herpesviruses on the one hand and EBV on the other hand.

A further example of the advantage of the IF test with counterstain is shown in Figure 2. Three different serum samples from a four year old child with an acute lymphoblastic leukemia with a suspected intercurrent CMV infection were tested by IF. These sera were sampled at day 1, 10 and 20 after onset of the first symptoms. By CF seroconversion could not be demonstrated because the second and third serum were anti-complementary. By IF it was easy to demonstrate seroconversion for CMV with a

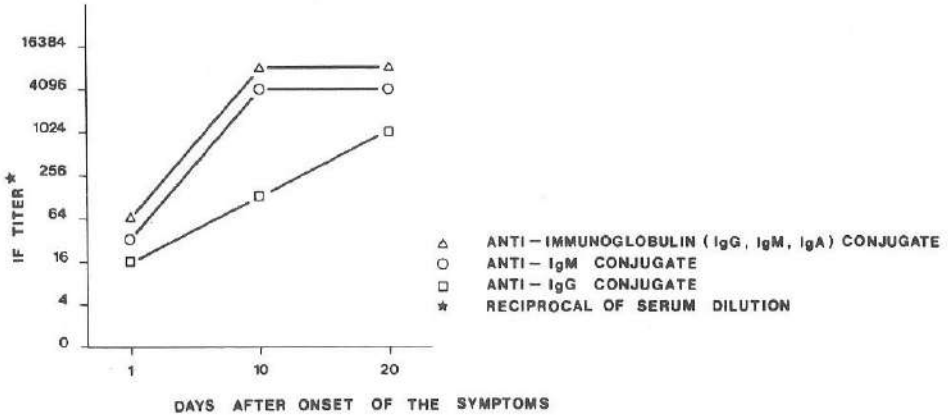

FIGURE 2. Titration for antibodies to CMV by IF in 3 sera from a 4 year old leukemic child, sampled on day 1, 10 and 20 after onset of the symptoms of an intercurrent infection.

primary IgM response and subsequent IgG response. The swine anti-human conjugate to IgG, IgM and IgA which is used in the regular IF procedure gives the same results as the conjugate to IgM alone.

Figure 3 shows the distribution of the IF titers in a large number of sera for the 4 herpesviruses. The total number tested varies from 135 for CMV to 360 for EBV-VCA. The sera are partly from healthy blood donors and laboratory personel, and partly from cancer patients attending the clinic and out-patient department of the Netherlands Cancer Institute. Several conclusions can be reached:

(1) The percentage of negative sera for the different viruses ranges from less than 1% for VZV up to 30% for CMV. This implies that there is a substantial difference in the percentage of infected adults for these 4 ubiquitous viruses. This assumption is only valid of a negative serum in the IF test actually means no previous infection with virus. The shape of the curves indicates a normal distribution of positives and points to that conclusion.

(2) There is a significant difference (t-value = 12.98: $P << 0.01$) in Geometrical Mean Titers (GMT) between VZV (1:59) and EBV (1:964), with HSV (1:107) and CMV (1:166) as intermediates. GMT's for HSV and CMV do not differ significantly from the GMT of VZV on the one hand and with the GMT of EBV on the other hand.

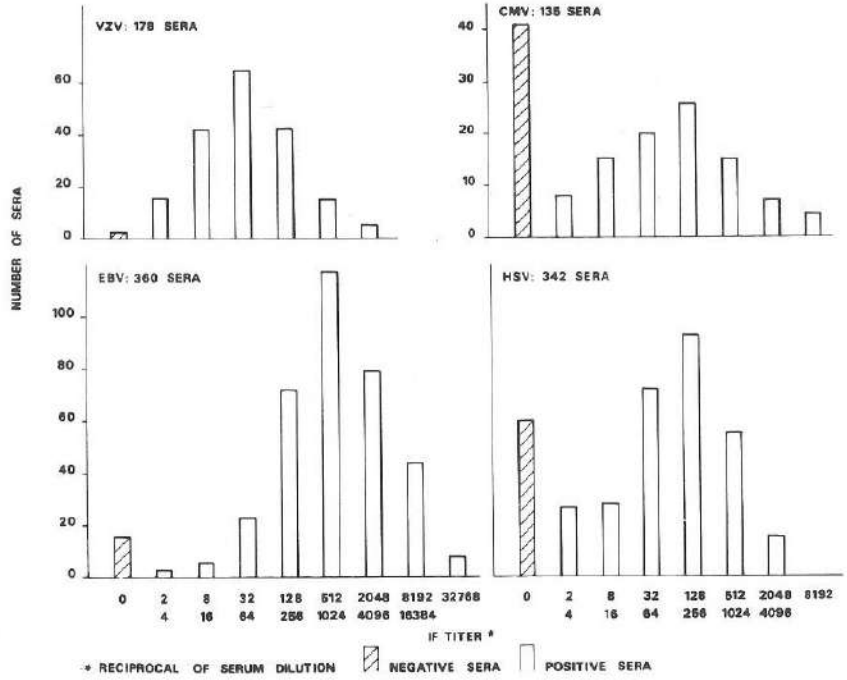

FIGURE 3. Histograms showing the titer distributions for HSV, VZV, CMV and EBV. The total number of sera tested ranges from 135 for CMV to 360 for EBV. The sera are from normal healthy individuals and from a wide variety of patients with neoplastic diseases.

Note that the highest antibody titers by the here described IF technique are 1:4096 for VZV and HSV, 1:16384 for CMV and no less than 1:32768 for EBV, indicative of the high sensitivity, *e.g.* as compared with previously described IF techniques.

A modification of the usual IF technique was necessary for the detection of EBNA (18). This modification implies the use of complement in combination with a conjugated anti-complement serum to detect this nuclear antigen: anti-complement immunofluorescence (ACIF) test.

A possible source of error in the detection of antibodies to this nuclear antigen is the presence of ANF. Since ANF shows a broad reactive antigen in the DNA of nuclei of cells of many species, it is sufficient and necessary to mix EBNA positive cells with EBNA negative cells, *e.g.* cells of murine origin. Since the murine cells were chosen to be of smaller size than the Raji cells, antibody titers to EBNA and ANF can be determined simultaneously. Table 2 shows some results of this titration in selected NPC sera.

This ACIF test using a mixture of cells includes 3 possibilities:

(1) EBNA positivity only: the 50% large cells show a fine granulated nuclear fluorescence,

(2) ANF positivity only: all cells show a coarse type of nuclear fluorescence,

(3) serum positivity for both EBNA and ANF: all cells show nuclear fluorescence of 2 different types. Since titers for EBNA and ANF are rarely

TABLE 2

COMPARISON OF ANTIBODY TITERS TO EBNA AND OF ANF TITERS IN SOME NPC SERA

Serum number	Date of sampling	EBNA titer*	ANF titer*
KCC 1244	06.11.1970	4096	256
KCC 1244	18.12.1970	4096	256
KCC 1245	28.07.1971	256	8
KCC 1245	13.08.1971	256	4
KCC 1249	05.05.1971	4096	64
KCC 1249	04.08.1971	2048	64

* Reciprocal of serum dilution.

The presence of ANF was previously shown by T.O. Yoshida, 1971 (74), and the sera were used by Reedman and Klein, 1973 (18). They were obtained from the Kenya Cancer Council (KCC) via Dr. G. Klein, Karolinska Institute, Stockholm, Sweden.

equal, 2 different endpoints can be recorded, one for 100% of the cells (ANF titer) and one for 50% of the cells (EBNA titer). In case 100% of the cells is positive at the endpoint the serum should be pre-absorbed with mouse cells. This theoretical possibility was not encountered in this study. It should be emphasized here that ANF present a complex of antigens, some of which might not crossreact among species.

The above described IF and ACIF techniques were used in sero-epidemiologic surveys of three types of malignancies:

(1) BL (34), (2) NPC, (3) HD.

Table 3 and Figure 5 show the statistical analysis and titer distribution in 20 sera from Tunesian patients with NPC and 20 age and sex matched healthy Tunesian controls. In contrast to BL, where antibody titers to EBV antigens as well as to VZV and CMV are elevated, in NPC elevations are only found to the 3 EBV antigens tested. Tables 4 and 5 and Figures 6 and 7 show the statistical analysis and titer distributions for 2 groups of HD (NS and MC) and their age and sex matched healthy controls. The age distribution of the patients whose sera were studied resembles the actual age distribution of HD in the Netherlands (35), *i.e.* a relatively high number in the age group of 20-29 years (Figure 4). The diagnosis of the 2 subgroups of HD (NS and MC) was confirmed according the Rye modification of the Lukes and Butler scheme (45) by one pathologist (Dr. J. van Unnik), the Netherlands Cancer Institute. The 2 other subgroups of HD in the Rye classification, the lymphocyte predominance and the lymphocyte depletion, yielded too little sera to make a statistical analysis valuable. The normal distribution of subgroups of HD is in correspondance with this classification, in which NS and MC form the two main classes (46).

TABLE 3

NASOPHARYNGEAL CARCINOMA
20 SERA FROM TUNESIAN PATIENTS WITH NPC AND FROM 20 HEALTHY AGE AND SEX MATCHED TUNISIAN CONTROLS

Antigen/ Virus	Negative sera		Geometrical Mean Titer		Student's t-value
	Tumor	Control	Tumor	Control	
EBV-VCA	0	0	1607	115	4.64*
EA	0	13	163	7	6.93*
EBNA	0	2	984	26	7.60*
HSV	2	1	15	47	2.57**
VZV	2	0	19	12	1.44
CMV	0	0	44	82	2.02

* Significant values (P = 0.01)

** Reversed significant value: the control group has higher titers than the tumor group.

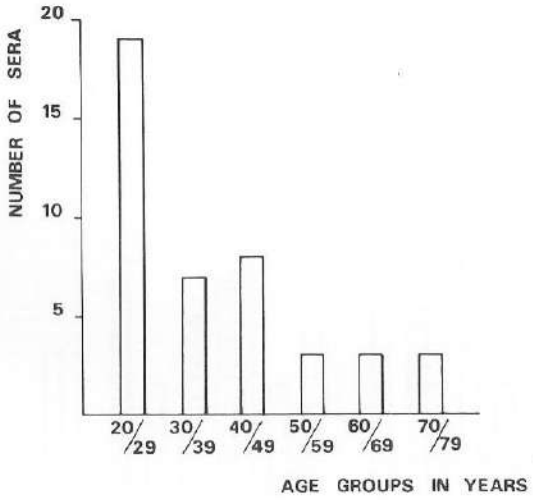

FIGURE 4. Age distribution of the two HD groups, *i.e.* MC and NS (see also Tables 4 and 5 and Figures 6 and 7).

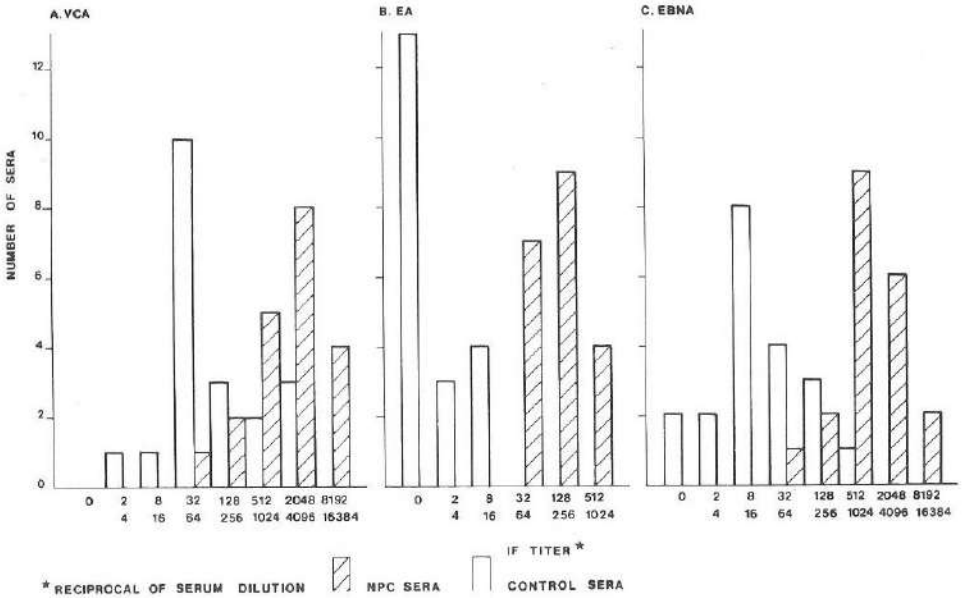

FIGURE 5. Histograms showing the titer distributions for VCA (A), EA (B) and EBNA (C) in 20 Tunesian patients with NPC and 20 age and sex matched Tunesian controls.

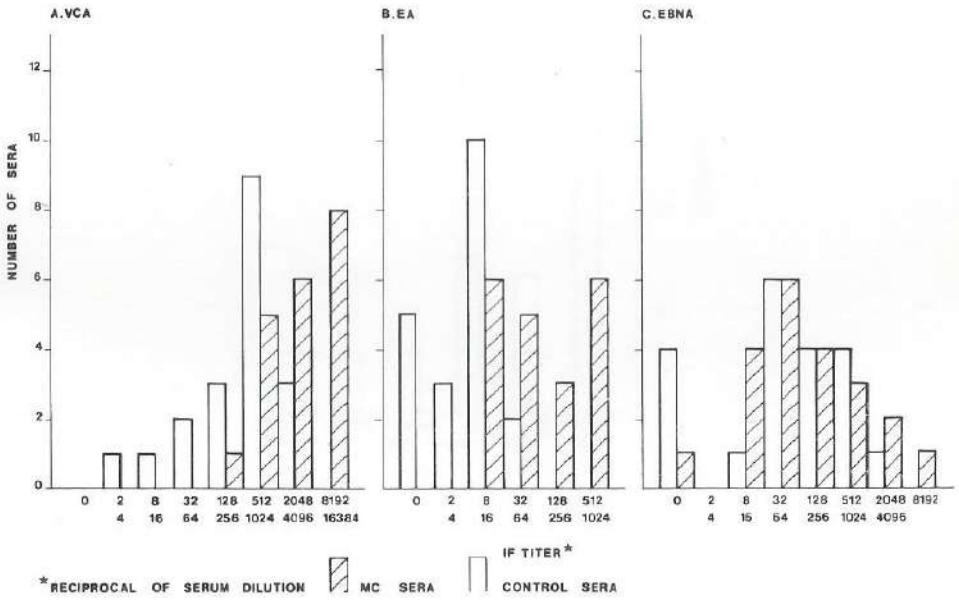

FIGURE 6. Histograms showing the titer distributions for VCA (A), EA (B) and EBNA (C) in 20 patients with the MC HD and 20 age and sex matched healthy controls.

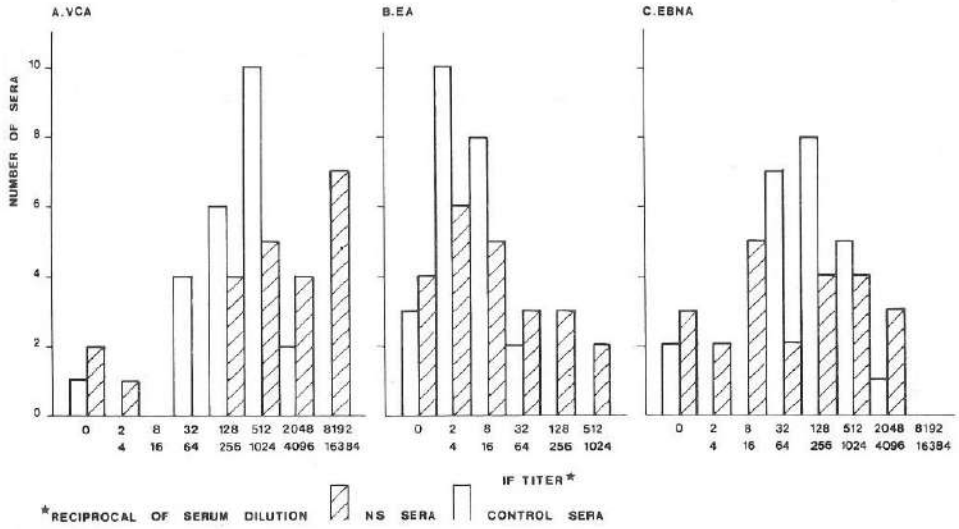

FIGURE 7. Histograms showing the titer distributions for VCA (A), EA (B) and EBNA (C) in 23 patients with the NS HD and 23 age and sex matched healthy controls.

TABLE 4

STATISTICAL ANALYSIS OF THE ANTIBODY TITERS TO HUMAN HERPESVIRUSES OF 20 PATIENTS WITH THE MIXED CELLULARITY HODGKIN'S DISEASE AND 20 AGE AND SEX MATCHED HEALTHY CONTROLS

Antigen/ Virus	Negative sera		Geometrical Mean Titer		Student's
	Tumor	Control	Tumor	Control	t-value
EBV-VCA	0	1	3673	396	4.46*
EA	0	5	111	13	4.76*
EBNA	1	4	186	169	0.14
HSV	5	3	71	55	0.19
VZV	1	0	74	55	0.60
CMV	4	5	256	52	2.27

* Significant values ($P \leqslant 0.01$)

TABLE 5

STATISTICAL ANALYSIS OF THE ANTIBODY TITERS TO HUMAN HERPESVIRUSES OF 23 PATIENTS WITH THE NODULAR SCLEROSING HODGKIN'S DISEASE AND OF 23 AGE AND SEX MATCHED HEALTHY CONTROLS

Antigen/ Virus	Negative sera		Geometrical Mean Titer		Student's
	Tumor	Control	Tumor	Control	t-value
EBV-VCA	2	1	1626	406	2.69*
EA	4	3	24	7	2.25
EBNA	3	2	128	198	0.72
HSV	3	3	104	48	1.11
VZV	0	0	81	37	1.75
CMV	6	9	84	67	0.32

* Significant values ($P \leqslant 0.01$)

While significant GMT differences between MC and the control group are found for both EBV-VCA and EA, no such difference was found for EBNA, HSV, VZV and CMV. In the case of NS a significant difference can only be found to EBV-VCA.

Compared to the picture for NPC one can draw the following conclusions:

(1) EBV-VCA titers in MC, NS and NPC are roughly 10x (significant), 4x (significant), and 10x (significant) higher in the tumor group versus the controls.

(2) EBV-EA in MC, NS and NPC are roughly 10x (significant), 3x (*not* significant) and 23x (significant) higher.

(3) EBNA in MC and NS is equal and in NPC roughly 38x (significant) higher.

Note that, although antibody titers to CMV in the MC of HD are about 5x higher than in the control group, this is not significant (P = 0.02). All NPC and HD sera of this survey were tested for ANF. Only a few NPC sera were positive.

DISCUSSION

The indirect immunofluorescence (IF) test with counterstain (43, 34, 19) and the anti-complement immunofluorescence (ACIF) test with counterstain (this paper and (44); the latter for coloured pictures) have proven to be an easy and useful method to determine antibody titers to the 4 human herpesviruses, *i.e.* herpes simplex virus (HSV), varicella-zoster virus (VZV), cytomegalovirus (CMV) and Epstein-Barr virus (EBV). The specificity and high sensitivity of this IF test could be demonstrated by the comparison with CF and ID.

In the case of herpes zoster and varicella it was observed previously (for review see Gold and Nankervis, 1973 (47)) that CF antibody titers to VZV are disappearing within 3 years after infection. This would be in contrast to infection with the other 3 herpesviruses, since here antibody titers remain remarkably constant and detectable throughout life. In our material less than 1% of the sera was negative for antibodies to VZV by IF. Comparing the sensitivity of the CF and the IF test (see Figure 1), it can be seen that IF titers up to and including 1:32 correlate with CF titers of less than 1:8 for the same sera. This would have resulted in negative CF results for VZV in approximately 44% of the sera in our test series. However, the distribution of IF titers to VZV points towards the fact that these results are reflecting the actual situation, that is a high incidence of virus-infected individuals, but with persistant and relatively low antibody level. This is furthermore supported by the fact that the GMT to VZV in our test series is lower (1:59) than to the other herpesviruses.

A routine procedure to detect a recent virus infection is to demonstrate a fourfold increase in antibody titer by CF. Anti-complementarity of sera, however, can make diagnosis troublesome, if not impossible. Using the IF test one does not encounter this problem. In fact one can distinguish the primary immune response in terms of IgM and IgG antibodies, as could be demonstrated for a patient with acute lymphoblastic leukemia with an intercurrent CMV infection. Hanshwa, 1968 (48), Langenhuyzen *et al.*, 1970 (49), and Caul *et al.*, 1972 (50) previously demonstrated the use of the IF technique for CMV in this respect. Schmitz and Scherer, 1972 (51) reported the same for EBV. It is obvious that the IF technique can also be used to determine fourfold increase in titers for (herpes-)viruses.

A complication of the comparison of IF titers with ID and CF titers is the possibility that these 3 tests detect antibodies to different antigens as suggested for CMV (52). Another complication relates to the possibility of crossreacting antigens between different herpesviruses (53, 54). The latter possibility seems unlikely (34).

With the IF test for antibody determinations for all 4 human herpesviruses, rather than IF for EBV and CF for HSV, CMV and VZV, it is possible to compare the relative immune response to these viruses. As shown in Figure 3 important differences exist in GMT's to these viruses. Most important is the difference in GMT's of VZV, HSV and CMV (1:59, 1:107 and 1:166 respectively) on the one hand and EBV (1:964) on the other hand. This might be a reflection of the contact of the antigens with the immune apparatus. Thus, while VZV and HSV are "hiding" in ganglion cells (55, 56 and for review on HSV see 57) and CMV in the salivary glands, polymorphonuclear leucocytes and sperm (for review see 58, 59, 60), EBV is present in lymphoid cells (61).

Sero-epidemiological studies for herpesviruses in HD have been carried out by several investigators, in Sweden (9), in the USA (10, 26, 27), in Denmark (25) and in the Netherlands (62). In the Swedish study (9) treated and untreated patients with HD of various histological groups, paragranuloma, granuloma and sarcoma (histological classification according to the criteria of Jackson and Parker, 1947) showed significant elevation in GMT to EBV-VCA in the granuloma and sarcoma group compared with age and sex matched controls. A correlation seemed to exist between high elevations and low number of lymphoid cells in the tumor. Levine *et al.*, (10), studying 63 treated and untreated HD patients, classified according to the Rye modification of the Lukes-Buttler scheme (45), essentially confirmed these findings. The lymphocyte predominant group (comparable with paragranuloma) did not differ significantly from the controls in their GMT for EBV-VCA, while the mixed cellularity showed the highest elevations. This study did not show elevated antibody titers by CF to HSV, VZV and CMV.

Henderson *et al.* (26), studying 142 patients with HD, again could confirm these findings, demonstrating also highest antibody titers in the mixed cellularity. While no elevation by CF was found to HSV and CMV, an indication for elevated titers to VZV was obtained from the comparison with 2 of 3 control groups. While in these early studies antibodies were determined to the viral capsid antigen of EBV only, subsequent studies by Hesse *et al.*, 1973 (25) and Henle and Henle, 1973 (27) also took in account EBV-EA. Hesse *et al.* (27) demonstrated an elevation of antibody titers to EA in sera of 15 untreated HD patients. They did not find titer fluctuations in the course of the disease in contrast to Levine *et al.*, 1971 (10). Henle and Henle, 1973 (27), in an extensive study including 489 patients with HD demonstrated elevations for EBV-VCA and EA titers. This study clearly

showed that not all HD patients are infected with EBV, suggesting no etiological role for this virus in all cases of this disease.

In the most recent study on the sero-epidemiology of HD, Langenhuyzen *et al.*, 1974 (62) confirmed earlier findings on elevations in titers to EBV-VCA in 25 Dutch patients with HD. They also claimed elevations in antibody titers to CMV, but the level of significance is chosen somewhat high (P = 0,02).

In our study antibody titers were not only determined to EBV-VCA and EA but also to EBNA. This resulted in the unexpected finding that in the mixed cellularity of HD the antibody pattern of the 3 EBV antigens, which is high to VCA and EA, but low to EBNA, is similar to the pattern observed in heterophile antibody positive IM (63), so far the only human disease known with certainty to be caused by EBV. This antibody pattern is in clear contrast to the patterns observed in NPC (see Figure 3) and BL (64), where antibodies to all 3 EBV antigens studied here are elevated. Since elevations to EBV-EA are found in various neoplastic and non-neoplastic diseases in humans and in EBV-induced malignancies in primates with high lymphoproliferative activity, one may conclude that such activity is the cause of the elevations in HD. However, as mentioned before, the lymphocyte poor forms of HD show the highest elevations. One must therefore look for another explanation of these results in HD. Such an alternative explanation can perhaps be found in a recent observation by Businco *et al.* as quoted by Klein, 1974 (65) about high antibody titers to EBV-VCA in combination with EA in 2 children with a congenital thymic immune deficiency. After thymus grafting in these children VCA titers fell to normal and EA titers virtually disappeared, as if restored T-cell function lowered the humoral immune response to EBV. Consequently, if this is the explanation for the elevated titers to VCA in combination with EA in the MC of HD, T-cell function might not be adequate in this type and different in this respect from the NS of HD. This view is strengthened by the recent observation of Johansson and coworkers (65a) that impaired T-cell functions in patients with HD correlate with elevated antibody titers to VCA. Significant elevations in antibody titers to the 3 herpesviruses other than EBV were not found in the 2 types of HD, although titers to CMV were higher in the MC- than in the controlgroup. The situation for CMV is quite comparable with that found by Langenhuyzen *et al.*, 1974 (62).

An interesting difference with regard to elevations in antibody titers to the 3 herpesviruses other than EBV exists between NPC and BL. As reported previously (44) antibody titers are significantly elevated to VZV and CMV in BL, but as shown here, no such elevations are found in NPC.

Heterophile antibody positive IM is caused by EBV (16, 63). When it was found that antibody titers to EBV are elevated in HD and in view of the fact that both diseases occur preferentially in young adults, several epidemiological studies were carried out to demonstrate a relationship between these diseases. Such retrospective studies (66, 67, 68) have contributed conflicting evidence in this respect. This is no surprise in view of the findings of Niederman *et al.*, 1970 (69), that seroconversion for EBV in adults may occur without any clinical symptoms.

Although at present HD is not thought to be caused by EBV, one must bear in mind that this is due (1) to the finding of the absence of EBV-specific nucleic acids or EBNA in this disease and (2) to the finding of EBV negative patients in all surveys mentioned before. The first negative finding, however, does not exclude positivity of a low percentage of tumor cells amongst a majority of non-malignant reactive cells. The second negative finding could be due to the heterogeneity of HD, one type of which could be caused by EBV. This would resemble the situation in IM, where the clinical picture can be caused not only by EBV but also by other viruses such as CMV (70). From infection experiments with primates it is clear by now that EBV can cause both an IM like disease (71) and malignant lymphoma and leukemia like diseases (72, 73).

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CHAPTER 5

Elevated immunofluorescence antibody titers to several herpesviruses in Burkitt's lymphoma patients: are high titers unique?

Elevated Immunofluorescence Antibody Titers to Several Herpesviruses in Burkitt's Lymphoma Patients: Are High Titers Unique? [1,2]

Frans Hilgers,[3] Andrew G. Dean,[4] *and* Guy de-Thé[5,6]

SUMMARY—Antibody titers for viral capsid antigens of all four human herpesviruses were measured by immunofluorescence in the sera of 16 Burkitt's lymphoma (BL) patients, 16 age- , sex- , and locality-matched controls, and 136 family members from the West Nile District of Uganda. Among family members, titers greater than 1:4 were found in 98% for herpes simplex virus (HSV), 86% for varicella-zoster virus (VZV), 100% for cytomegalovirus (CMV), and 94% for Epstein-Barr virus (EBV). Titers in patients averaged ≈2 logs (fourfold) higher than those in matched controls for EBV, VZV, and CMV ($P=0.001$); titers for HSV were only slightly higher in cancer patients. The mothers of patients had somewhat higher EBV titers ($0.05 \leq P \leq 0.01$) than the mothers of controls, but no other differences between patient and control families were found. By immunofluorescence, a method which apparently has not been used for all four human herpesviruses in BL patients, the patients had elevated antibody titers not only to EBV but also to CMV and VZV. The elevated titers to three of the four human herpesviruses were not due to serologic cross reactions.—J Natl Cancer Inst 54: 49-51, 1975.

HIGH TITERS OF ANTIBODY to Epstein-Barr virus (EBV) were found by the fluorescent antibody (FA) test in a high percentage of Burkitt's lymphoma (BL) patients (*1*, *2*), and EBV is involved in most etiologic hypotheses about BL (*2–5*). Few serologic studies of other herpesviruses were reported in BL patients (*6*, *7*), and apparently none measured antibodies to all four viruses by the indirect immunofluorescent method (*1*, *8*).

In a recent field study in West Nile, Uganda (*9*), the clinical histories of many BL patients suggested that herpes simplex infection had occurred shortly before onset of the tumor. FA titers for herpes simplex virus (HSV), varicella-zoster virus (VZV), cytomegalovirus (CMV), and EBV were therefore measured in sera from the patients with BL and from controls and their families. Not only EBV but also CMV and VZV titers were higher in patients than in controls.

MATERIALS AND METHODS

Sera were collected in West Nile from 16 BL patients, from 16 controls of the same age and sex, and from 136 other individuals in patient and control families. Controls were selected by random numbers from tax lists in the same subcounty as the patients (*9*). Blood specimens were allowed to clot before overnight storage at 4° C. The sera were stored at −20° C and then placed in liquid nitrogen for shipment and further storage.

Antibody to EBV was tested at the IARC with standard methods for the viral capsid antigen (VCA) test (*1*, *8*) and fourfold serum dilutions beginning at 1:10. Antibody against the other three viruses was measured at The Netherlands Cancer Institute by a similar indirect immunofluorescence method (*10*) with a 1:40 dilution of fluorescein-conjugated swine antihuman immunoglobulin obtained from Nordic, Tilburg, The Netherlands (in preparation). The antigens were as follows: HSV type-2 obtained from a vaginal specimen and grown in RK13 cells; VZV (isolate 69-115) from a zoster patient, grown in diploid human embryonic lung fibroblasts; and a CMV isolate, "Reints," grown in the same strain of cells. The VZV and CMV strains were provided by Dr. K. W. Slaterus, Wilhemina Gasthuis, Amsterdam, The Netherlands, who identified them by their cytopathology and reactions with known antisera in complement-fixation tests. Infected cells were mixed with an equal number of uninfected cells before being applied to the microscope slides, dried overnight, and then fixed in acetone for 10 minutes before storage at −20° C. Sera were diluted in fourfold steps beginning with 1:4. HSV type-1 and -2 gave similar results in this test, and a single antigen (type-2) was therefore used for convenience. Patient and control sera identified only by number were tested together; the clinical histories were unknown to those performing the test.

RESULTS

All patients and controls had titers of 1:16 or more to HSV and CMV and 1:10 or more to EBV (1 of the 16 patients was not tested for EBV). Two patients and five controls had no titer (<4) against VZV; the others had titers of 1:4 or above. Table 1 indicates the geometric mean titers (GMT) of all groups tested. Titers were ≈4 times (2 logs) higher in patients than in controls for VZV, CMV, and EBV (table 2). The differences were statistically significant at the $P \cong 0.001$ level for all three by the Student's *t*-test. HSV titers were slightly higher in patients than in controls. The mothers of patients had higher titers for EBV than the mothers of controls. In case and control families, statistically significant titer differences were found only between cases and their controls, not between their mothers, fathers, and siblings, and the differences were almost equal in magnitude for VZV, CMV, and EBV.

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TABLE 1.—*GMT of matched BL patients and controls with their families for four human herpesviruses*

Test group	HSV	VZV	CMV	EBV, VCA
BL patients	224.8(16)[a]	32(16)	378.1(16)	249.1(18)
Controls	167.6(18)	9.7(18)	133(18)	55.5(18)
Patient families	141(93)	17.2(93)	191.4(93)	65.3(94)
Control families	147(90)	14.1(90)	206.3(90)	54.8(88)

[a] Number of sera tested *in parentheses*.

TABLE 2.—*Differences in FA titer between matched BL patients and control individuals for four human herpesviruses*

Subjects	HSV	VZV	CMV	EBV
BL patients+controls	0.38[a] (16)[b]	2.00(16) ($P \cong 0.001$)	1.75(16) ($P \cong 0.001$)	1.87(15) ($P \cong 0.001$)
Siblings	−.45(38)	0.13(38)	−0.61(38)	−0.12(39)
Mothers	.75(12)	.83(12)	.50(12)	1.35(10)
Fathers	.33(12)	1.08(12)	−.58(12)	−0.09(11)

[a] Average $= (\log \frac{\text{case titer}}{\text{control titer}})$. This is equivalent to the average number of "tubes" difference in titer for twofold dilutions.

[b] Numbers of pairs tested *in parentheses*.

DISCUSSION

Numerous studies have been done on EBV titers of groups of patients and controls with the FA method, and elevated EBV titers have been associated with BL (*1*, *2*), nasopharyngeal carcinoma (*11*), infectious mononucleosis (*12–14*), Hodgkin's disease (*15*, *16*), lupus erythematosus (*17*), and Izumi fever (*18*), though only in the first three does the association seem solidly established. A few investigations have reported antibody levels for other herpesviruses in the same sera but have used complement fixation rather than FA for this work (*7*, *16*). One report mentions elevated complement-fixation titers for VZV in Hodgkin's disease (*16*). The present study is the first report of FA titers for all four human herpesviruses in a group of BL patients and controls. The elevation of CMV and VZV titers and those for EBV suggests that testing of other serum collections with the FA technique might produce valuable information and shed some light on the meaning of a "high" titer to a herpesvirus—the central unanswered question which underlies EBV serology.

Our results might be due to chance, to variability of the FA test, or to factors peculiar to the ecology of West Nile. FA tests in the EBV field have shown that variability up to two or even three dilutions can be obtained from one testing to another, depending, among other factors, on the antigen batch (type of cell line and proportion of immunofluorescent cells) (*19*). When CMV, VZV, and HSV antibodies were tested, similar variability was observed. However, the titers were obtained in tests in which coded patient and control sera were interdigitated and the sera were from a small but carefully matched series of cases and controls. The elevation of FA titers for VZV, CMV, and EBV can be looked for in larger groups of BL patients and controls and by other laboratories.

Cross-reactions among the human herpesviruses in the FA test could produce high titers to several viruses in the same serum. Other workers have not found such cross-reactions (*8*) except for a partial serologic relationship between HSV and VZV (*20*), though a macroglobulin reacting with CMV was detected in the serum of some patients with other herpesvirus infections (*21*). Our results for the 68 children, 15 years and under, in control families tested the cross-reaction hypothesis. The titer for each virus was plotted against the titer of one of the other three viruses in the same serum. The results for each possible pair of viruses showed a complete scatter; there was no evidence of relationship between a high titer for one virus and high or low titers for any of the others. For each possible pair of viruses, sera could be found with high titers for one virus (HSV-256, VZV-128, EBV-160, CMV-512) which had low titers for the other (HSV and VZV-4, EBV-10, CMV-32). Finally, 20 sera from patients with nasopharyngeal carcinoma and 20 controls, which were tested by our laboratories together with the present BL series with the same antigens, showed highly elevated titers for EBV but not for the other human herpesviruses. These findings have been extended and will be reported separately (in preparation). Serologic cross-reaction does not seem a reasonable explanation of the findings.

Dean et al. (*9*) previously suggested, from a clinical-epidemiologic survey, that lesions resembling herpes simplex infection occur with unusual frequency just before the development of BL tumor. Since these

herpetic-type lesions have healed by the time the patients came to the hospital, it was impossible to attempt isolation of HSV from these lesions; it was hoped that serology would yield clues about the cause of the oral ulcers and unilateral conjunctivitis described by the patients (*9*). However, since all the patients had HSV titers of 1:64 or higher and all the controls had titers of 1:16 or more, HSV infection can neither be ruled in nor out. Patients with a history of herpes-like lesions did not have higher HSV titers than did controls or the other patients. Douglas and Couch (*22*) showed, however, that patients with recurrent herpes infections do not necessarily have high neutralizing antibody titers or rises in titer with recurrences, and there is no reason to believe that FA titers would be different in this regard. Hence the failure to find "elevated" HSV titers in our BL patients does not prove or disprove the clinical hypothesis of an association between BL and HSV. The hypothesis that the tumors are caused by an interaction between EBV and another herpesvirus would be difficult to test in a population in which nearly everyone is infected by several herpesviruses at an early age.

The association between BL and EBV is not only supported by seroepidemiologic data, but also by the regular presence in African BL tumor cells of EBV fingerprints, i.e., EBV genomes (*23*, *24*) and EBV-specific nuclear antigen (*25*). The presently reported FA titers for other herpesviruses should be completed by other tests directed against early antigen, membrane antigen, and soluble antigen of CMV, VZV, HSV-1, and HSV-2. BL biopsy specimens should be examined for virus genomes other than EBV. The on going prospective seroepidemiologic study of BL in the West Nile district of Uganda (*26*) would provide the proper material from BL cases and controls to perform such seroepidemiolgic and molecular virologic studies.

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SAMENVATTING

De inleiding tot dit proefschrift wordt gevormd door een overzichtsartikel, waarin de oncogene capaciteiten van herpesvirussen worden beschreven. Achtereenvolgens wordt aandacht besteed aan:

1) Lucké herpesvirus (kikker)
2) Marek's herpesvirus (kip)
3) herpesvirus Saimiri en herpesvirus Ateles (Nieuwe Wereld Apen)
4) Epstein-Barr virus
5) herpes simplex virus type 1 en type 2
6) cytomegalovirus

De laatste drie zijn menselijke herpesvirussen.

Het doel van het experimentele werk dat in de 5 publicaties van dit proefschrift beschreven wordt, is geweest het verbeteren van bekende immunologische technieken voor het aantonen van antilichamen tegen en antigenen van humane herpesvirussen en deze vervolgens te kunnen toepassen voor kwantitatieve sero-epidemiologische studies. De belangrijkste techniek die gebruikt werd, was conventionele immunofluorescentie, direct of indirect. Deze kon verfijnd worden door het gebruik van een mengsel van geïnfecteerde en ongeïnfecteerde cellen en door het tegenkleuren van de cellen met Evans' blauw. Voor alle vier de herpesvirussen, herpes simplex virus (HSV), varicella-zoster virus (VZV), cytomegalovirus (CMV) en Epstein-Barr virus (EBV) kon een in essentie gelijksoortige techniek gebruikt worden. Hiermee is een gevoelige, snelle en nauwkeurige bepaling van de antilichaamtiter tegen deze virussen mogelijk.

Terwijl wij met het practische werk voor dit proefschrift bezig waren, ontdekte Reedman in Klein's laboratorium, dat met de anti-complement immunofluorescentie (ACIF) techniek, in tegenstelling tot de conventionele immunofluorescentie (IF) techniek, het EBV-geassocieerde kernantigeen (EBNA) aangetoond kan worden. In samenwerking met Reedman en Klein bleek het mogelijk de methode verder te verfijnen door middel van de tegenkleuring met Evans' blauw. Titerbepalingen zijn zo nauwkeurig, dat het mogelijk werd semi-kwantitatieve absorptie tests voor de verschillende antigenen van EBV uit te voeren. De IF techniek is hiervoor het meest aangewezen, omdat door de specifieke intra-cellulaire localisatie van de antigenen deze herkend kunnen worden.

Samenvatting van publicatie 1:
Immunofluorescence and anti-complement immunofluorescence absorption tests for quantitation of Epstein-Barr virus-associated antigens. Int. J. Cancer, 15, 566-571, 1975.
Beschreven worden immunofluorescentie absorptiemethoden waarmee kwantitatieve bepaling en differentiatie van EBV geassocieerde antigenen (virus capside antigeen, VCA, vroeg antigeen,

EA en EBNA) in celextracten mogelijk zijn. EBNA was aanwezig in alle cellijnen (producerend en niet-producerend), waarin het EBV genoom aanwezig is, terwijl VCA en EA alleen in producerende lijnen gevonden worden. Al deze antigenen waren afwezig in lymphoide cellijnen (MOLT-4), waarin het EBV genoom ontbreekt, zowel als in leukemie-cellen van het perifere bloed. Met behulp van deze technieken kon aangetoond worden dat de antigenen van de verschillende cellijnen identiek zijn.

Omdat het niet mogelijk is het membraan-antigeen (MA) van EBV te kwantificeren op gefixeerde cellen en de membraan immunofluorescentietest voor dit antigeen op levende cellen practische problemen oplevert, werd de Radio-Iodine Elution (RIE) test gebruikt voor de relatieve kwantificering van MA op levende cellen. Dit leidde tot de onverwachte ontdekking, dat in deze test niet alleen MA wordt gekwantificeerd, maar ook de intracellulaire antigenen VCA en EA en waarschijnlijk ook EBNA.

Samenvatting van publicatie 2:
Radioiodine-labeled antibody elution (RIE) for detection of EBV determined antigens: competition for specific labeled antibodies using suspended antigens. Clin. Immunol. Immunopathol., 2, 542-555, 1974.
EBV antigenen werden bepaald en gekwantificeerd in menselijke lymphoblastoide cellijnen met behulp van specifiek met radioactief jodium gemerkte antilichaam uitwassing (RIE). De hoeveelheid specifiek uitwasbaar antilichaam blijkt toe te nemen bij stijgende doses van EBV, 2-3 dagen na super-infectie van gevoelige cellijnen. Verhoging van de antilichaamconcentratie bij een constant celaantal laat een liniaire toename zien van uitwasbaar, zich specifiek aan de producerende cel bindend antilichaam. Verhoging van het aantal cellen bij een constante antilichaamconcentratie leidt ook tot een liniaire toename van de specifiek uitwasbare antilichamen. Oplossingen van antigenen bereid uit bevroren en ontdooide cellen, werden gebruikt in competitie met antigenen op de doelwitcellen voor de antigeen bindende plaatsen van de gemerkte antilichamen. Er werden zowel experimenten uitgevoerd waarbij ter competitie oplossingen gebruikt werden van cellen die alle EBV antigenen bevatten, als experimenten waarbij de competitie beperkt was tot EA.

Er werd daarom een methode ontwikkeld om door middel van directe immunofluorescentie aan te tonen dat "levende" cellen inderdaad toegankelijk zijn voor specifieke antilichamen.

Samenvatting van publicatie 3:
Detection of Epstein-Barr virus-determined intracellular antigens in unfixed cells with labeled antibodies. J. Nat. Cancer Inst., 53, 949-955, 1974.
EBV antigenen werden bepaald in menselijke lymphoblastoide cellijnen met behulp van de RIE en IF tests. Voorafgaande onderzoekingen met de RIE test waarbij suspensies van ongefixeerde cellen werden gebruikt, wezen uit dat niet alleen MA maar ook de intracellulaire antigenen VCA en EA werden aangetoond. In dit onderzoek werden met de RIE test zowel de R en D subtypes van EA ontdekt in cellen, waarin EA was geïnduceerd door 5 Iodo-2'-deoxyuridine. Om te bepalen waaraan de specifieke antilichamen in deze niet gefixeerde celsuspensies binden, werden met fluoresceine gemerkte, voor EBV specifieke antilichamen gebruikt. De gemerkte antilichamen bleken de cel binnen te dringen en zich daar aan de intracellulaire antigenen VCA en EA te binden. Wanneer ze gemengd werden met niet gefixeerde celsuspensies, konden deze antilichamen direct zichtbaar gemaakt worden in de cel na fixatie en tegenkleuring met Evans' blauw. Er bleek geen verschil te zijn in het percentage gekleurde cellen, wanneer antilichamen werden toegevoegd voor of na fixatie. Geconcludeerd kon worden dat antilichamen ongefixeerde cellen kunnen binnendringen en zich daar specifiek binden aan EBV antigenen.

Tenslotte werd het practische nut van beide gemodificeerde immunofluorescentie technieken voor sero-epidemiologisch onderzoek aangetoond.

Samenvatting van publicatie 4:
An immunofluorescence technique with counterstain on fixed cells for the detection of antibodies to human herpesviruses; antibody patterns in patients with Hodgkin's disease and nasopharyngeal carcinoma. Intervirology, in press.
Een indirecte immunofluorescentie (IF) test op gefixeerde cellen met tegenkleuring werd beschreven voor de humane herpesvirussen: herpes simplex virus type 1 en 2 (HSV-1 en HSV-2), VZV, CMV en EBV. Vergelijking met immunodiffusie (ID) in het geval van HSV-2 en met ID en complementfixatie (CF) voor VZV en CMV toonde de specificiteit en grote gevoeligheid van deze IF test aan. Een modificatie van de anti-complement immunofluorescentie (ACIF) test voor EBNA werd geïntroduceerd. Hierdoor bleek het mogelijk tegelijkertijd een antilichaamtitratie uit te voeren voor dit kernantigeen en voor de anti-nucleaire factor (ANF). IF titers tegen de verschillende virussen in onze testseries

waren veel hoger voor EBV (1:964), dan voor CMV (1:166), HSV (1:107) en VZV (1:59). Sero-epidemiologisch onderzoek voor deze virussen bij patiënten met de ziekte van Hodgkin in Nederland liet het volgende patroon zien: (1) in de nodulair scleroserende vorm van de ziekte van Hodgkin is er een viervoudige verhoging in antilichaamtiter ten opzichte van VCA maar niet van EA en EBNA en (2) in de gemengd cellige vorm van de ziekte van Hodgkin wordt een tienvoudige verhoging ten opzichte van VCA en ook EA maar niet van EBNA gevonden, vergeleken met de controle groepen. Deze patronen bij de nodulair scleroserende en de gemengd cellige vorm van de ziekte van Hodgkin verschilden van het patroon bij het nasopharynxcarcinoom, waar een verhoging in antilichaamtiter werd gevonden tegen alle drie de EBV antigenen. Antilichaamtiters tegen HSV, VZV en CMV waren niet verhoogd bij patiënten met de ziekte van Hodgkin en met het nasopharynxcarcinoom.

Samenvatting van publicatie 5:
Elevated immunofluorescence antibody titers to several herpesviruses in Burkitt's lymphoma patients: are high titers unique?
J. Nat. Cancer Inst., 54, 49-51, 1975.
Antilichaamtiters tegen de menselijke herpesvirussen werden bepaald met de IF test in de sera van 16 patiënten met het Burkitt's lymphoom, 16 op leeftijd, geslacht en woonplaats geselecteerde controlepersonen en 136 familieleden uit het West-Nijl district van Oeganda. Titers hoger dan 1:4 werden gevonden onder familieleden in 98% voor HSV, 86% voor VZV, 100% voor CMV en 94% voor EBV. Titers voor EBV, VZV en CMV waren in patiënten gemiddeld 4x hoger dan die in de geselecteerde controlepersonen ($P = 0{,}001$); titers voor HSV waren slechts licht verhoogd bij de patiënten. De EBV titers waren bij moeders van patiënten iets hoger ($0{,}05 \leqslant P \leqslant 0{,}01$) dan bij moeders van controle personen, maar andere verschillen tussen patiënten en controle families werden niet gevonden. Met behulp van IF, een methode die bij patiënten met het Burkitt's lymphoom nog niet eerder werd gebruikt voor het bepalen van antilichamen tegen alle vier de menselijke herpesvirussen, blijkt dat de patiënten verhoogde antilichaamtiters hebben, niet alleen ten opzichte van EBV, maar ook van CMV en VZV. De verhoogde titers tegen 3 van de 4 menselijke herpesvirussen zijn niet te wijten aan serologische kruisreacties.

STELLINGEN

1. Hoewel de ziekte van Hodgkin op grond van vroeger sero-epidemiologisch onderzoek in verband wordt gebracht met Epstein-Barr virus (EBV), is nu op grond van dergelijk onderzoek met meer antigenen van EBV ernstige bedenking te uiten tegen een causaal verband tussen beide.

2. De vroege diagnostiek van het anaplastische carcinoom van de rhinopharynx (nasopharynxcarcinoom) kan verbeterd worden door serologisch onderzoek met de verschillende EBV-antigenen.

3. Bij de beoordeling van het effect van radiotherapie op het nasopharynxcarcinoom, is het volgen van de antilichaamtiters tegen de verschillende EBV-antigenen onmisbaar.

4. In veel gevallen van Burkitt's lymfoom buiten de endemische gebieden in Afrika wordt deze pathologisch-anatomische diagnose ten onrechte gesteld, doordat immunologisch en virologisch onderzoek naar het voorkomen van EBV bij deze lymfomen ontbreekt.

5. De recente ontdekking van EBV-DNA in tumorcellen van patiënten met een (angio-)immunoblastaire lymfadenopathie rechtvaardigt een uitbreiding van onderzoek naar andere door herpesvirussen geïnduceerde tumoren bij de mens.

   G.W. Bornkamm et al. Int. J. Cancer, 17, 177-181 (1976).

6. Tot nog toe wordt de veronderstelling dat een idiopathische perifere facialisparalyse (Bell's palsy) veroorzaakt zou worden door een latente herpes simplex virusinfectie, niet ondersteund door voldoende sero-epidemiologisch en virologisch onderzoek.

7. De huidige vaccinatie tegen recidiverende herpes labialis heeft geen wetenschappelijke basis en houdt bovendien het risico in van het introduceren van oncogene varianten van herpes simplex virus.

8. Het gebruik van een "vomerflap" bij de primaire sluiting van een gehemeltespleet verstoort de ventro-caudale uitgroei van de bovenkaak.

9. Als de voortvarendheid waarmee de actie "Geven voor Leven" gevoerd werd ook zou worden toegepast bij de honorering van de ingediende researchprojecten, waren inmiddels vele miljoenen nuttig besteed en zou een positieve bijdrage ter vermindering van de werkeloosheid, ook onder academici, zijn geleverd.

10. Voorvechters van acties tegen luchtverontreiniging die zelf blijven roken, handelen in strijd met hun doelstellingen.

Amsterdam, 9 september 1976 F.J.M. Hilgers

Waar de krachten te kort schoten, is niettemin de goede wil te prijzen.

Ovidius (43-18 voor Chr.)