Immunological studies on Burkitt's lymphoma

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A FEW years ago, it became clear, in the progress of experimental tumor immunology¹⁻⁷ that all virus-induced tumors carry a common, group-specific transplantation antigen, capable of inducing rejection reactions in syngeneic hosts. The question arose whether it would be possible to utilize this fact for the search after possibly similar, virally determined transplantation antigens in tumors of unknown etiology, particularly human. Our attention turned to Burkitt's lymphoma for three reasons:

- (a) its postulated viral etiology⁸;
- (b) clinical observations⁹⁻¹¹ strongly suggesting that host-defense reactions may play an important role in this disease and decisively influence the outcome of chemotherapy;
- (c) among the virally induced experimental tumors, lymphomas lend themselves particularly well for the demonstration of virally determined common antigens, partly because it is easy to prepare free-cell suspensions, with a high frequency of viable cells, and partly because humoral antibodies are more easily demonstrable, as a rule, against surface antigens carried on lymphoma cells than on large carcinoma or sarcoma cells.

The first question we asked appeared quite straightforward: was it possible to obtain by testing the reactivity of live Burkitt lymphoma (BL) cells, with patients' sera, and in comparison with appropriate controls, evidence that would indicate the existence of characteristic cell-membrane-associated antigens, in analogy with the virally induced murine leukemias and, if so, could this information help to elucidate the etiology of the disease, as well as the possible role of host defense reactions that may influence its clinical course? To approach this problem, we chose the technique that was most

sensitive in the experimental leukemia studies,^{12, 13} viz. membrane immunofluorescence with viable target cells.¹⁴ The findings, summarized briefly in the following chapters, essentially confirmed the expectations, but they have also led to many unexpected observations and raised new dilemmas. Some of them may serve to exemplify the problems encountered during the transition from the experimental to the human situation.

Studies on BL biopsy cells

During the first phase of this work, fresh BL biopsy cells were exposed to the sera of BL patients and various other donors and we were looking for attached immunoglobulins by the indirect membrane fluorescence technique.^{101, 102, 110} The sera of BL patients reacted more frequently than African control sera from donors with other neoplastic or non-neoplastic diseases. The possibility that the reactivity of the BL sera was due to isoantibodies became unlikely when it was found that autochthonous serum-cell combinations gave positive reactions in five of six cases where this could be tested. It turned out, furthermore, that the most regularly positive sera have been derived from patients whose tumors have gone to total regression after chemotherapy. For this reason, the autochthonous target cell was frequently unavailable from highly positive serum donors. To exclude isoantibodies, such sera were tested in parallel series against lymphoma cells and normal bone marrow cells derived from the same allogeneic BL donor. Lymphoma cells, but not bone marrow cells, reacted regularly in such tests, thus increasing the probability that the reactivity of the BL serum-cell combinations could not be simply due to the presence of isoantibodies. This was further reinforced by the finding that lymphoid cells of normal donors and of donors with different types of leukemias and other lymphoreticular diseases also failed to react.

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While this was encouraging, further studies on the specificity of the reaction were hampered by the great variability of the biopsy preparations. One major source of the difficulty was the variable degree of immunoglobulin coating on the surface of the biopsy cells. This coating was detected by direct membrane immunofluorescence with conjugated anti-immunoglobulin reagents. It could be of two basically different kinds: IgM and/or IgG, showing not only a difference in class specificity but also a difference in behaviour in relation to the course of the disease.^{15, 16} In the cases where the cell surface reacted with anti-IgM conjugates, reactivity was usually expressed on 100% or nearly 100% of the cells. When such cells were converted into established lines *in vitro*, their 'IgM-ring' was maintained during long-term propagation. The membrane-IgM reactive lines did not secrete IgM into the medium.¹⁷ Preliminary characterization of the reactive substance in membrane fractionation experiments¹⁸ indicates, at least as far as one cell of this type is concerned, that 7S-size IgM subunits with μ - and κ -chain specificities are integrated into the cell membrane. Conceivably, this is the neoplastic variety of a normal lymphoid cell that incorporates molecules of this type into its plasma membrane as part of its normal differentiation. Lymphoid cells of this type have been postulated to play an important role in immunological memory and/or delayed hypersensitivity.¹⁹ The lymphoma cells may represent the neoplastic variant in the same way as myeloma cells project normal immunoglobulin-secreting plasma cells into a magnified, neoplastic image. The phenomenon is not exclusive for BL; a Swedish case of chronic lymphatic leukemia has been found with the same cellular characteristics.²⁰ Quantitative comparisons between different lymphoma lines of this type showed¹⁸ that the different lines carry very different amounts of IgM-kappa reactive material on their membrane. Although an extensive search has been made on more than sixty biopsies and derived lines, so far no line has been found that would carry other types of membrane-associated immunoglobulins than IgM-kappa.

Whatever the nature of the cell that carries membrane-associated immunoglobulins, it is important to note in relation to the present discussion that this property has always behaved as a cell marker when repeated biopsies were taken from the same patient. If it was present on the cells of a given tumor, it was maintained unchanged in the course of repeated biopsies; if it was absent, it remained absent. It was also maintained following successful heterografting of a membrane-IgM-positive BL cell to the rat.²¹ The IgG coat behaved quite differently. It was rarely present on untreated BL biopsy cells, but it tended to appear if the tumor persisted in spite of treatment.

It accumulated following a recurrence that was presumably due to the selection of a tetraploid, probably immunoresistant, BL cell variant.^{16, 22} 'Self-enhancement', i.e. the accumulation of 'blocking' antibodies that prevent the access of immune lymphoid cells⁷ is an obvious possibility.

Whether these considerations are realistic or not, the changing pattern of IgG coating with time and its failure to persist on derived *in vitro* lines,^{15, 23} indicate that it is due to coating from the outside, unlike the membrane-associated IgM, that appears to represent a special type of production from the inside.

The presence of preformed immunoglobulin on the cell surface may interfere with the indirect membrane immunofluorescence reaction and, when present in subliminal degree, it probably explains some of the variability encountered when biopsy cells are used as targets. In order to avoid this variability, we started looking for more standardized target cells and turned to established culture lines.

Experiments with established tissue culture lines

A number of BL-derived lymphoblastoid cell lines growing in stationary suspension cultures were tested against BL sera that reacted regularly with BL biopsy cells, and were free of demonstrable isoantibodies.²⁴ The pattern appeared strange but interesting. Four BL-derived lines gave positive membrane immunofluorescence reactions in the indirect test after exposure to the reference serum 'Mutua' (derived from a BL patient in long-term regression) whereas three BL-derived lines were negative. Eight control lines derived from various leukemias and, in one case, from a normal donor, were negative as well. At first, we could not understand this pattern. A clue was obtained, however, when these results were compared with the reactivity of the same cell lines in the Henle test²⁵ known to detect EB-viral (probably nucleocapsid) antigens. In carrier cultures, these antigens are present in a small frequency of the cells, as a rule. These cells show degenerative features and, when simultaneous immunofluorescence and electron microscopy are performed^{26, 27} turn out to contain herpes-type Epstein-Barr (EB) virus²⁸ particles. The first comparison of the membrane and EBV test revealed²⁹ that the four membrane-positive lines contained EBV-antigens in more than 1% of the cells whereas the membrane-negative lines were either EBV-negative or contained a very small frequency of positive cells (less than 1%).

This suggested that the membrane antigen detected by this reference serum may be determined by the genome of the EB virus. More conclusive evidence was obtained in a prospective study.²⁹ Fourteen new lines were established from biopsies received from Nairobi, and the frequency of EBV-positive and of

membrane-reactive cells was determined in parallel, on coded specimens, at two different laboratories. The same relationship was found as in the preliminary retrospective study: only the lines that carried a relatively high 'EBV-load' showed a positive membrane antigen reactivity. In the reactive lines, the frequency of membrane-positive cells was approximately ten times higher than the frequency of EBV-positive cells. The biopsies from which the lines were derived were membrane-positive, but EBV-negative, as a rule. EBV reactivity appeared during the first week in culture. This suggests that the production of the viral nucleocapsid antigen is suppressed in the tumor cell *in vivo*. The suppressive factor could be antibody, but there are many other possibilities. Another curious observation was that repeated establishment of parallel lines from the same patient, derived from successive biopsies, led to lines with fairly similar EBV levels, whereas lines derived from different patients were quite different.³⁰ This suggests that the viral 'load' per cell, or the activatability of the virus, or both, are characteristic for the individual tumor. Since the membrane-associated IgM marker, mentioned above, and another study with G6PD-isozymemarkers³¹ strongly indicate that the BL process has a clonal origin, this may reflect the virus-cell relationship that characterizes a particular clone. This is also suggested by the closely similar levels of EBV-DNA hybridizable cellular DNA in repeated biopsies taken from multiple tumors from the same BL patient.³²

The postulate that the membrane antigen is determined by the EB virus, was directly confirmed when it was found that it can be induced to appear in EBV-negative lines by the admixture of heavily irradiated EBV-carrying cells,¹¹¹ or by infection with EBV concentrates.^{33, 34} In the infected cells, membrane antigen appeared after 20–24 hr. DNA-inhibitors such as cytosine arabinoside (Ara C) or IUDR did not prevent its appearance, whereas puromycin inhibited it completely. It behaves, in other words, like an 'early' product of the viral genome, not requiring viral DNA synthesis. In this respect, it shows certain parallels with membrane and T antigens found in experimental oncogenic DNA virus systems.^{35, 36, 6}

Although the relationship between EBV and the membrane antigen was clarified by these studies, this applies only to the EBV-carrier cultures *in vitro* and it must be kept in mind that similar compelling evidence is lacking about the connection between the membrane antigens detected on the biopsy cells and the virus, although there are strong indications that the biopsy cells probably express the same membrane antigen as the carrier cultures.³⁷

The antigenic components entering the EBV-determined membrane and the intracellular nucleo-

capsid complex, respectively, differ with regard to immunological specificity. By absorbing sera that reacted with the membrane and the intracellular EBV complex as well, with large numbers of intact, viable membrane antigen-positive cells it was possible to remove the membrane-reactive antibodies, with only a minor reduction in the anti-EBV titer.³⁸ Moreover, some sera could be found with antibodies against the membrane antigen, or the EBV antigen, but not both. Although such 'discordant' sera were in minority, their existence is in line with the immunological distinctness of the two antigen types.

Further analysis of the two antigen systems revealed⁹⁵ that both the membrane and the intracellular antigens must be regarded as antigen complexes, with several distinct subcomponents. Sera that contain antibodies against several subcomponents of the intracellular EBV complex also tend to carry, as a rule, several antibody components against various parts of the membrane-antigen complex, but the relationship is not absolute and many combinations can be found. Patients with large, persisting tumors frequently had a larger number of serum antibody components against both antigen complexes than sera from healthy, EBV-positive individuals, or convalescent sera from donors after infectious mononucleosis, or sera from BL patients whose tumors have gone to long-term regression following chemotherapy.

The nature of the membrane antigen, particularly its specification by the viral or the cellular genome remains to be clarified. Recently, indirect evidence has accumulated suggesting that it may represent a viral envelope component. The ability of different sera to neutralize an artificial EBV infection of EBV-negative culture lines (such as Raji or 6410) was related to the titer of membrane-reactive antibody, and not to the anti-EBV titer.³⁹ This was particularly apparent when a series of sera were tested that were discordant with regard to their anti-EBV and membrane reactivity. In another series of tests, the sera of rabbits immunized with EBV concentrates were able to block the membrane-antigen reaction specifically.⁴⁰ This indicated that the membrane-antigen was present in the immunizing material, either as a constituent or as a contaminant of the viral particles.

It has been shown that herpes simplex virus (HSV) is capable of including new membrane-antigens in the cells it infects.⁴¹ Viral mutants with different envelope characteristics induce different membrane changes, in a way that closely parallels their envelope properties.⁴² It has been concluded that the appearance of viral envelope material is responsible for the changes in the cell membrane. Presumably, the virus changes the cellular membrane in order to facilitate the process of its own envelopment. In view of the parallel between EBV neutralization and membrane-

reactive antibody levels, it is conceivable that the EBV-associated membrane-antigen represents viral envelope material as well. This is of interest, because, for HSV, a relationship has been demonstrated between the changed 'social behavior' of infected cells and their membrane modifications after exposure to different mutants of HSV.⁴² The understanding of the role EBV-induced membrane changes may play for cell behavior may elucidate the relationship between this agent and the neoplastic diseases with which it is most regularly associated.

Disease-related serological patterns

These can be discussed at two levels: (a) the relationship between EBV-associated serological reactivity and the clinical and pathological diagnosis; (b) changes in EBV-related serological patterns during the clinical course of EBV-associated diseases.

Concerning (a), it can be first stated that the serological anti-EBV reactivity, as determined by the Henle test²⁵ is extremely widespread in all human populations. If the level of significant reactivity is set at a 1 : 10 serum dilution, as customary,⁴³ the large majority of adult populations is EBV-positive. It may be questioned whether the 10–15% negatives (with titers <10) are real or spurious. Specific antibodies may occur at titers below 10 and may be missed, due to the various test artefacts that arise at high serum concentrations⁴³. On the other hand, whereas some of the <10 'negatives' may hide specific reactivity, at least *part* of them must be *real* negatives in the biological sense. A prospective study⁴⁴ has shown that EBV-positive young adults are protected from infectious mononucleosis, whereas a significant proportion of the 'EBV-negatives' (i.e. <10) developed the disease and became EBV-positive, in the course of a 2–4 years observation period.

The causal relationship between EBV and at least one form of infectious mononucleosis⁴⁵ is most clearly established by this prospective study. If this is accepted, it immediately leads to the question whether EBV plays any role in other diseases and particularly the neoplastic diseases with which it is most regularly associated.

The serological patterns that are now known can be evaluated in different ways. In the Henle type anti-EBV test, Burkitt lymphoma (BL) and nasopharyngeal carcinoma (NPC) are distinguished by outstandingly high anti-EBV titers, so far unparalleled amongst other lymphoproliferative diseases and other carcinomas of the head and neck region.^{43, 46–48} The geometric mean anti-EBV titer of BL patients was eight times higher than in various control groups. There were no significant differences between control sera collected from areas with a high or a low incidence of Burkitt's lymphoma.

With the exception of a few moribund cases, low (<1 : 80) anti-EBV titers were very rare among BL patients and there are no histologically confirmed cases with negative (<1 : 10) titers. Occasionally, long-term regression cases tended to show falling titers after some years, but this was by no means the rule. The serological behavior of BL and NPC is also exceptional in the precipitin test developed by Old, Oettgen *et al.*,^{49, 50} performed against a soluble antigen extracted from the EBV-carrying P3J line. NPC sera from African and American patients were positive in 85–87% and 59% of the African BL sera gave positive precipitin reactions. Other neoplasms, including lymphoproliferative diseases and carcinomas of different kinds gave a much lower incidence of positives, with the exception of chronic lymphatic leukemia and lymphosarcoma that came close to the reactivity of the BL sera. Two distinct precipitin lines (B and P) could be identified regularly but there was no obvious disease-related difference between the two.

The antibodies against the EBV-associated membrane-antigens can be most easily evaluated by the blocking of the direct membrane fluorescence reaction, obtained with specific reference conjugates.^{51, 52} When the Mutua conjugate was used, already referred to in the previous paragraph, Burkitt's lymphoma⁵³ and nasopharyngeal carcinoma sera⁴⁷ showed a high blocking activity, whereas the sera of normal African controls, Burkitt patients' relatives and African tonsillitis patients showed mostly negative reactions, even though occasional positives were encountered. Head and neck tumors, other than Burkitt's lymphoma and nasopharyngeal carcinoma were also largely negative but occasional highly positive sera have been encountered in this material as well. The difference between the regularly high-reactive African or Chinese nasopharyngeal carcinomas and the predominantly low-reactive Indian hypopharyngeal and oropharyngeal carcinomas was particularly remarkable.⁴⁷

In a 'tripartite' study, the anti-EBV (Henle) test, the blocking of direct membrane fluorescence and the precipitin reaction have been compared with 151 coded sera.⁵³ There was a clear overrepresentation of BL and NPC sera within the 'triple-high' reactivity group and they were virtually absent from the 'triple-low' group. The opposite was true for the two main groups of control sera, derived from healthy relatives of BL patients and from donors with head and neck tumors other than BL and NPC.

One interesting question concerns the relationship between geographical localization and serological reactivity. Nasopharyngeal carcinomas are more easy to evaluate in this respect, since they represent a clear pathological entity and are not readily

confused with other conditions. The EBV-associated serological reactivity of African, Swedish, French, Chinese and American cases was uniformly high and appeared to be characteristic for the anaplastic or poorly differentiated type.^{46, 47, 50}

The evaluation of Burkitt's lymphoma outside Africa presents a more difficult problem, because the pathological picture alone does not permit a sharp distinction against other lymphomas. The combined clinical and pathological picture has readily recognizable features in the high-endemic areas, but they are less characteristic in other regions and the classification becomes more arbitrary. If one nevertheless examines the data on the serological behavior of 'Burkitt-like' lymphomas outside Africa, it appears that the results are partly in line with the African Burkitt lymphomas,^{53, 54} and partly differ from the African cases, i.e. have no distinctively high EBV-associated reactivity (defined as high anti-EBV titer and/or high membrane-blocking index) and thus resemble ordinary lymphosarcomas rather than 'true' Burkitts. This picture cannot be interpreted meaningfully at present since serology cannot serve as the basis for classification if the problem is to decide whether non-African cases have an African-Burkitt-like serology or not; the argument becomes circular. Speculatively at least, one may nevertheless consider the possibility that the non-African Burkitt-like cases are heterogeneous. Some of them would be 'true Burkitts', i.e. have the same etiology as the African cases, whereas others would be different and comparable to 'ordinary' lymphosarcomas. Whether this classification can be based on the EBV-associated serological patterns will, of course, depend on the question whether the relationship of the EB virus to Burkitt's disease is of an essential or of an accidental nature.

(b) Another approach to the study of disease-related EBV patterns is to follow the antibody titers against the various EBV-associated antigens horizontally, during the course of 'EBV-associated' diseases, such as BL and NPC. For comparison, one may choose EBV-positive individuals with more or less related neoplastic diseases that are not regularly associated with high anti-EBV titers. Studies of this type are now becoming feasible; some preliminary information is already available. In BL, the indirect membrane test, performed with biopsy cell targets has indicated at an early stage^{101, 102} that the most highly reactive sera can be found in patients whose tumors have gone to long-term regression. Later, when the more specific and sensitive blocking of direct membrane fluorescence replaced the indirect test as the main method to detect antibodies against the EBV-associated membrane-antigen complex on established culture lines, it turned out^{52, 53, 55} that nearly all histologically confirmed African BL sera

have a high blocking activity, i.e. show a complete or nearly complete cross-reactivity with the reference conjugate. The few exceptions have come from moribund patients. This monotonously uniform blocking activity, obtained with the undiluted sera hides large quantitative differences, however. When compared by serial titration against the same reference conjugate, the blocking titer of various BL patients' sera (taking a blocking index of 0.5 as the endpoint) can vary between 1 : 1 and 1 : 600.⁵⁵ In the individual patient, the titers may change considerably in the course of the disease, but, as a rule, they remain within the same order of magnitude: most changes are restricted to relatively few dilution steps up or down and the patients can be therefore classified into groups of low, medium and high reactivity.

Our preliminary findings indicate that the blocking titer differences between patients, as well as the horizontal changes, are influenced by a number of factors. In the course of rapid and extensive tumor growth, antibody levels probably fall, due to adsorption to tumor cell membranes. When the patient receives chemotherapy and the tumor regresses, there is often an increase in titer. At first sight, this may seem paradoxical, in view of the immunosuppressive effect of chemotherapy. It is known, however, that chemical immunosuppression inhibits new primary antibody responses against antigens administered after the drug, but is much less efficient against immune reactions established before treatment.

An increase in blocking antibody titers was particularly apparent in BL and NPC patients who received local radiotherapy,⁵⁶ including cases where therapy did not lead to complete tumor regression. In view of the fact that X-irradiated tumor cells are relatively good immunogens in experimental systems⁵⁷ this is of considerable interest. It may also be relevant that in EBV-carrier cultures with a relatively low membrane-antigen reactivity, X-rays can induce the appearance of the membrane-antigen on a large fraction of the cells.⁵⁸

In BL patients with recurring tumors that continue to grow in spite of chemotherapy, blocking antibody levels that have fallen to low levels at or around the time of recurrence can rise again.¹⁶ Subsequently, the lymphoma cells become coated with IgG, as a rule, if the tumor persists. It is conceivable that such cells represent immunoresistant variants, similar to what has been found in experimental systems.⁵⁹ This is supported, indirectly at least, by the history of two patients whose tumors recurred after several years of total regression and contained a high frequency of near-tetraploid cells,²² in contrast to more than twenty other BL biopsies examined,⁶⁰ with a shorter clinical history, that were all in the near-diploid range. Tetraploid cells can

frequently outgrow host responses that efficiently reject diploid cells of the same lineage.⁶¹

The immunoglobulin coat acquired by the tumors that persist in spite of therapy may be the equivalent of enhancing antibody or of blocking antibody in Hellström's sense.⁷ This is not necessarily an alternative to the possibility that membrane-reactive antibodies may have a growth-inhibitory action but rather another facet of the same complex picture. An antibody that has cytotoxic or growth-inhibitory properties against immunosensitive cells may exert an enhancing effect (i.e. protect the target cell against the cell-mediated immune response) when it interacts with an immunoresistant cell without killing it. In addition, different antibodies no doubt differ; some can be cytotoxic and others enhancing towards the same target cell. In the course of chemotherapy that falls short of a total tumor kill, and the subsequent regrowth of the residual tumor with more antigen release and antibody binding, the immunosensitivity of the tumor cell population and the killing vs. enhancing power of the antibody population must obviously change in a complex way. This would require a multicomponent experimental analysis, that is not yet within reach.

In addition to the changes in membrane-reactive antibody levels brought about by the tumor itself (i.e. changes due to absorption, antigen release, effect of tumor growth on the immune response, etc.), the antibody titer may change for other, tumor-unrelated reasons, and this may, in turn, influence tumor growth. This possibility has been brought into focus by the history of a BL patient¹⁶ who was in total tumor regression for a period of 4½ years and subsequently developed widespread abdominal metastases. Her membrane-reactive antibody level, determined by the blocking test, fell markedly more than 6 months prior to recurrence, at a time when there was no reason to suspect the presence of any metastases. When the abdominal recurrence became manifest 6 months later, the membrane-reactive antibody level was still low, and the tumor cells were not yet coated with IgG. In the course of the subsequent 2 months, the serum antibody level increased again and the lymphoma cells became IgG-coated. This secondary sequence of events decreases the probability that the fall of the antibody level that preceded recurrence by 6 months was due to absorption to an as yet cryptic tumor, because, in that case, a period of slow tumor growth would have followed during the subsequent 6 months period, and the secondary increase in antibody level, as well as the coating of the lymphoma cells with immunoglobulins would be expected to have occurred in the interim, already appearing at the time of clinical recurrence. Indirect as this reasoning is, it has nevertheless raised the question whether a fall in antibody levels

may be sometimes the cause, rather than merely the consequence, of tumor recurrence, and whether it could act by facilitating the outgrowth of 'dormant' neoplastic cells.

There is some preliminary evidence indicating that the antibodies against the soluble EBV-associated antigens detected by immunoprecipitation show a different disease-related pattern, appearing at the time of progressive tumor growth and frequently absent in patients whose tumors are in complete regression.^{16, 53} Although there are numerous exceptions to this, a relationship of this type appeared clearly when the horizontal history of the patient already mentioned above, was followed during long-term regression and subsequent recurrence.¹⁶ This may also explain why high anti-EBV titrated NPC sera are more frequently precipitin-positive than BL sera with comparably high titers: in NPC, the serum material is mainly derived from patients with residual or progressively growing tumors, while a collection of BL sera include regressor and regressor sera as well.

Recently, Henle *et al.* have described yet another EBV-associated antigen, designated as EA (early antigen),⁹⁶ detected in EBV-infected Raji or 6410-cells. The sera of some EBV-positive donors, but not of others, contained antibodies against it. EA precedes the classical Henle-type 'EBV-antigen' during the infectious cycle; the latter has been renamed as 'VCA' (viral capsid antigen). The appearance of EA is readily prevented by puromycin but not by DNA inhibitors.³⁴ The behaviour of EA is thus not unlike what has been mentioned above for MA, with one important exception: whereas MA is compatible with continued cell growth and DNA synthesis, EA inhibits host macromolecular synthesis, as shown by a combination of immunofluorescence and autoradiography⁹³ and thus probably signals the entry of the cell into the lytic cycle. In contrast to EA, VCA is dependent on DNA synthesis and therefore probably represents a 'late' viral product.

A study of anti-EA titers in BL patients with known anti-VCA titers showed that tumor-bearing patients have anti-EA antibodies more frequently and in higher titers than patients whose tumors have gone to total regression. Also, whereas both BL, NPC and acute infectious mononucleosis (IM) patients have anti-EA antibodies, anti-VCA-positive healthy donors only exceptionally show any anti-EA reactivity at all and, if they do, they have much lower titers than tumor-bearing patients. In BL, a relationship could be demonstrated between the presence of anti-EA antibodies and the probability of recurrence. In untreated patients and in patients tested at the time of regression, high anti-EA titers showed a certain correlation with a poor prognosis.⁶⁴

Although there is thus a superficial similarity

between the disease-related patterns shown by the anti-EA antibodies and the precipitating antibody levels against the soluble antigen of Old *et al.*, mentioned above, it is also clear that the two antigens are different. Although the reactivity against them is correlated in the majority of the sera compared, a sufficient number of 'discordant sera' exist to prove that the antigenic specificities are not identical.⁶⁴ Both represent intracellular, and probably non-structural, early proteins, however.

The tumor-related presence of precipitating and of anti-EA antibodies is reminiscent of some DNA-virus-induced experimental tumor systems, and particularly the case of antibodies against 'T-antigens'. In polyoma, SV40 and adenovirus-induced tumors, it is the 'tumored hamster', i.e. the host of non-virus producing, T-antigen positive tumors that tends to develop antibodies against T-antigens. The antibody levels usually fall when the tumor is removed or rejected and eventually disappear. T-antigens are intracellular, like the soluble antigens in the present system. They are also 'early' components of the viral cycle, independent of DNA synthesis³⁵ and they are regularly present in transformed cells. In the former respect (independence of DNA synthesis) they are similar to, in the latter respect (compatibility with cell multiplication) they are different from the EA antigens. As far as the T-antigens are concerned, it is not known whether they are released from growing tumor cells by some kind of secretory process, or are only liberated from dead and dying cells. A release of the EA antigen may well be related to an abortive virus cycle, perhaps induced in tumor cells on their way to necrosis, i.e. under circumstances where the virus cycle cannot proceed to completion. The frequent absence of anti-T and anti-EA antibodies after tumor removal is in sharp contrast to the membrane-reactive antibodies, which tend to remain high, or even increase following rejection in experimental systems and as far as this has been studied, probably in the BL system as well. This may be related to the continued presence of 'dormant' tumor cells with a preserved, virally determined, foreign membrane antigen, held in check, but not killed by the host immune mechanism.

Further clarification of the relationship between the dynamics of antibody formation against different EBV-determined antigens and the clinical course of the 'high-EBV-associated diseases', such as BL and NPC, may be helpful in elucidating important virus-tumor-host relationships, particularly if compared with EBV-positive sera from patients with other tumors that are not characterized by a regularly high EBV-association.

Nucleic acid hybridization studies

Recent work of zur Hausen *et al.*,^{32, 65} demon-

strating the presence of cellular DNA capable of specific hybridization with purified EB-virus DNA is of considerable interest in the present context and may help to understand the serological results. By this technique, it was shown⁶⁵ that the BL-derived Raji line does contain EBV-DNA, in spite of the fact that none of the three EBV-associated systems MA, EA and VCA, can be demonstrated by immunofluorescence.⁶⁵ Since VCA and EA are not demonstrable in BL biopsy specimens³⁰ either, as a rule, although MA antigens are,²⁴ a recent study concerning the presence of EBV-DNA-hybridizable cellular DNA in biopsy specimens is of interest. Thirteen biopsies from ten BL donors all showed the presence of EBV-DNA, with 2-26 genome equivalents per cell.³² In three donors who yielded double biopsies, there was a remarkable agreement between the two tumors examined, although they were located in different anatomical sites (2-2, 7-8 and 21-26 approximate EBV genome equivalents per cell). In ten nasopharyngeal carcinoma biopsies, EBV-DNA was present in all, with 1-19 genome equivalents per cell. In ten tumors of other, miscellaneous kinds, taken from EBV-seropositive donors, there was no demonstrable EBV-DNA. The same was true for a number of other controls, including various established cell lines of human origin, cytomegalovirus-infected lines, Marek's tumor in chickens etc.

These studies thus show, in agreement with the serology, that there is a more intimate EBV-tumor association in BL and NPC than in other tumors occurring in anti-EBV seropositive donors.

Implications and dilemmas

Four main dilemmas arise from this pattern of findings; they are interrelated but all have their specific aspects. They can be briefly stated as follows:

(a) *The etiological dilemma.* Can the occurrence of distinctive, tumor-associated antigens give any clues about the etiology of the disease?

(b) *The problem of neoplastic behavior.* Are the changes in the composition of the cell membrane, or other cellular organelles, as reflected by the appearance of new antigenic specificities, fundamentally involved in the neoplastic behavior of the cell, or, in other words, does the unresponsiveness of the cell to growth control depend on the change in composition or structure that is revealed by the immunological tests?

(c) *The therapeutic problem.* Can any of the immunological reactions now identified serve to measure the patient's reactivity to his own tumor, in connection with various therapeutic procedures, including attempts at immunotherapy?

(d) Are there any *prevention* approaches in sight?

Concerning the *etiological dilemma*, it is a useful point of departure that all virally induced experi-

mental tumors share the same antigen, as long as they are induced by the same virus, at least as far as the transplantation-type, membrane-associated antigens are concerned. The reverse, the assumption of a common viral etiology on the basis of common antigens found in tumors of unknown origin is not necessarily justified, however. It has been shown⁶⁶⁻⁷⁰ that virally induced new antigens can be made to appear by superinfecting normal cells or tumors of unrelated etiology with oncogenic and even with some non-oncogenic viruses. The only difference between this secondary 'antigenic conversion' and the primary event that occurs in direct relation to tumor induction, is the lesser stability of the former, particularly in immune hosts,⁴ as well as a more irregular association between antigen and tumor, depending on the accidental nature of superinfection.

As discussed above, high anti-EBV titers and high antibody levels against EBV-associated membrane antigens and soluble antigens are regularly associated with at least two neoplastic diseases: Burkitt's lymphoma and nasopharyngeal carcinoma. For nasopharyngeal carcinoma, it is clear that this serological pattern is independent of geographical or ethnic origin. A similar situation may exist for Burkitt's lymphoma, but the lack of reliable criteria by which the identity of the disease can be established outside the endemic areas and distinguished from ordinary lymphomas makes a similar evaluation of the non-African cases more difficult.

It is important to stress that the main difference between BL and NPC and other normal or neoplastic serum donor categories investigated is not EBV-positivity, nor the occurrence of high-titered reactions in occasional donors, since such donors may be found in most other categories as well, but the *regular and consistent* association of high-titered reactions according to all three tests. Looking at it from this angle, BL and NPC are unique. One may question, however, whether this angle can be justified or, more specifically, what it implies.

As a starting point, we may take the convincing demonstration that EBV is causally related to at least one form of infectious mononucleosis.^{44, 45} This form afflicts EBV-seronegative adolescents, as a rule, is frequently positive for heterophile antibodies and is regularly accompanied by seroconversion to anti-EBV positivity. As indicated by a prospective study,⁴⁴ anti-EBV individuals are apparently protected from the disease.

The serological screening of many different human populations also showed^{33, 43} that there is another, 'early' seroconversion to anti-EBV positivity, culminating around 4 years of age, and particularly frequent in low socio-economic groups. This early infection does not lead to infectious mononucleosis or any other disease entity so far recognized.

Viewed against this background, the relationship of EBV infections to BL and NPC may be considered in terms of the following alternatives:

(a) The virus that causes infectious mononucleosis is also responsible for these two tumors; if this is true, intrinsic or extrinsic co-factors have to be postulated to explain the malignant conversion (the '*co-factor hypothesis*').

(b) Different virus subtypes are responsible for the different clinical entities (the '*multiple virus hypothesis*').

(c) The virus is a relatively harmless inhabitant of lymphoid tissues, although it may cause temporary proliferation (mononucleosis) under certain conditions. When lymphoid tissues proliferate for other reasons, e.g. in malignancies due to other, unrelated causes, the virus travels along as a passenger, with increased antigen production and high-titered antibody formation as a result. This '*passenger hypothesis*' is the logical analogue of the 'antigenic conversion' of established tumors by etiologically unrelated viruses, discussed above. In view of the high regularity of association, a requirement for a particular trophic relationship between EBV and the target (lymphoid) tissue may be added in the present case.

The passenger hypothesis cannot be excluded at present, but it appears less likely in view of the fact that lymphoproliferative diseases other than BL and anaplastic carcinomas other than NPC do not show a regular high-titered EBV-association. This statement includes malignancies occurring in the same or closely adjacent anatomical areas, such as reticulum cell sarcoma, lymphosarcoma, etc., and carcinomas that arise in or close to the tissues of the Waldeyer ring, such as the hypopharynx, oropharynx, the tonsil, base of the tongue, soft palate, etc. Carcinoma of the maxilla is a possible exception, but larger groups remain to be investigated. Hodgkin's disease represents a very interesting case in itself. Recently it has been found that the sarcomatous form, i.e. the lymphocyte-poor type with the worst prognosis, shows a high anti-VCA and anti-MA reactivity, quite comparable with BL and NPC, whereas the lymphocyte-rich and relatively more benign paraneoplastic form is low-reactive in both tests and thus resembles the control material.⁴⁶ The granulomatous form was intermediate, both with regard to histological type and serological reactivity. This means, as far as the serology is concerned, that it represents a mixture of high and low-reactive cases. Whereas it is thus possible that EBV plays some special role in the etiology of Hodgkin's sarcoma, the inverse correlation with lymphocytic predominance would not be in line with a simple passenger hypothesis.

None of this reasoning excludes the passenger

hypothesis conclusively, of course, but quite a number of *ad hoc* assumptions have to be made to maintain it in face of this evidence. One would have to postulate some specific trophic relationship between the virus and the kind of lymphocyte that gives rise to BL and is particularly abundant in NPC, that would not apply to the lymphoid cells that proliferate in the various other malignancies, used as controls. No valid objection can be raised against such a hypothesis, but it appears rather far-fetched in view of the fact that EBV-carrying blastoid cell lines can be regularly isolated from EBV-positive individuals, including donors with lymphoreticular malignancies of the 'control' type, i.e. diseases that do not show a consistently high EBV-positive serology. The sarcomatous form of Hodgkin's disease is also very hard to explain.

The possibility that EBV acts together with some co-factor in causing neoplastic disease or, to phrase the same thesis differently, that it acts by increasing the likelihood of neoplastic transformation brought about by other factors, has been recently proposed by Burkitt⁷¹ as far as the etiology of BL is concerned. In order to fit the geographic distribution of the disease with an ubiquitous virus, Burkitt proposed that an insect-transmitted co-factor is responsible for the malignant manifestation and specified it as chronic holoendemic malaria. This was based on the absence of BL from certain areas where malaria control has been enforced for some time and its presence in adjacent regions where malaria control was not regularly practiced.

It may be agreed that interactions between viruses and other agents, capable of stimulating the proliferation of a target tissue may lead to malignant transformation in experimental systems where neither the virus nor the other agent is oncogenic *per se*.⁷² Since chronic malaria exerts a strong proliferative stimulus on the RES, Burkitt's modified theory is reasonable, although it may be objected that the same picture would result from the transmission of *any* etiological factor or co-factor mediated by the appropriate insect, and this includes other viruses. Recently, some preliminary evidence has been obtained concerning the frequency of the sickling trait in BL patients,⁷³ however, that indicates a possible role of malaria in the causation of the disease.

The third possibility, the multiple virus hypothesis, implies the existence of closely related but biologically different EBV-viruses with differences in their oncogenic power and their target tissue preference. In light of the information derived from experimental oncogenic viruses, this is a realistic alternative as well. As far as leukemia viruses of the RNA type are concerned, it will be recalled that prior to the discovery of the interference test for avian leukosis virus classification,⁷⁴ it was not

possible to distinguish by morphological or immunological means between the viruses that were responsible for the different lympho- and myeloproliferative diseases or for fowl sarcoma. It is now known that the avian leukosis-sarcoma virus group has many closely related members; some induce solid tumors with highly distinctive properties, others are responsible for myeloid or erythromyeloid leukemia, or lymphomatosis, and still others cause no recognizable disease at all. A closely similar development can be noticed in the murine leukosis-sarcoma field. The Friend, Moloney, Rauscher, Gross, Kaplan, Rich, Graffi, Mazurenko, etc. agents are similar antigenically and indistinguishable by ultrastructure, but they induce distinct and characteristic clinical and pathological disease entities, specific for the viral agent.⁷⁵ In the DNA field, a possibly relevant example is the series of herpes simplex mutants, studied by Roizmann and his colleagues.⁴² Although this is not known to be an oncogenic system, it is important that different viral mutants induce different membrane changes in infected cells and, concomitantly, the cells are altered in their 'social behaviour' in ways that are characteristic for the virus mutant. Although a lytic virus obviously cannot transform its targets, the cellular changes are nevertheless concerned with intercellular relationships. Conceivably, other, non-lytic viruses of the same family might induce membrane changes compatible with cellular viability and reproductive integrity, and a social behaviour changed in the direction of disobedience to growth regulation—or, in other words, neoplasia. It may be recalled in this connection that the agents of at least two neoplastic diseases, Marek's neurolymphomatosis in the chicken⁷⁶ and Lucké's carcinoma in the frog⁷⁷ were recently identified as herpes-type viruses. A simian lymphoma is also probably due to a herpes-type virus (*Herpesvirus saimiri*).⁷⁸

The immunological tests so far performed on EBV-associated antigens, including those referred to in the previous sections, are not necessarily competent to reveal finer differences between closely related but biologically different agents with cross-reactive or overlapping antigenic components. A preliminary study of the membrane-antigens carried on EBV-positive lymphoblastoid cell lines derived from BL and NPC did not show any difference in the reactivity patterns,⁷⁹ but this may simply reflect the insufficient discriminating ability of the test.

Further studies are needed to distinguish between these possibilities. In order to narrow down the passenger hypothesis, more extensive tests are desirable on tumor categories where occasional sera gave high EBV-associated reactivity but only limited samples have been tested. It is also desirable to conduct nucleic acid hybridization studies on the corre-

sponding tumors. More refined analytical methods are needed for the attempts to dissect different virus variants. The recent developments in the herpes simplex field suggest that biochemical studies on viral envelopes and altered cell membranes may be particularly rewarding.

As far as the sero-epidemiology of EBV-infections is concerned, it has to be emphasized that there were no significant differences between low and high BL-endemic areas with regard to the distribution of anti-EBV titers in relation to age;⁴³ a comparison of titers in normal populations is therefore unlikely to elucidate the possible role of this virus in Burkitt's lymphoma. A prospective sero-epidemiological study may be more rewarding. It may be relevant in this connection that BL is essentially a childhood disease, with a peak incidence between 4 and 7 years. This fact, together with the clinical and serological evidence indicating a relatively high antigenicity in the autochthonous host would speak for a short latency period during the oncogenic process. In experimental systems, highly antigenic tumors arise with short latency periods, as a rule; or to put it in other words, highly antigenic tumors cannot escape rejection unless they grow out rapidly after their inception.^{1, 3, 80}

If this reasoning is essentially correct and the latency period of BL is relatively short, a prospective sero-epidemiological study may be decisive. In the populations of risk within the high endemic areas, as in other populations, there is a relatively small minority of EBV-negative children and another, even smaller minority with high anti-EBV titers. The majority consists of low-titered positives.⁴³ The question is whether BL develops in one of the two minority groups or at random and irrespectively of anti-EBV titer. Provided that a sufficiently large number of sera could be collected and stored under appropriate identification, tests may become feasible on pre-disease sera from individuals who develop Burkitt's lymphoma within a few years' time. It might be objected that the sensitivity threshold of the anti-EBV test may be too high (1 : 10) and a fraction of anti-EBV positive sera may be classified as false negatives. Since more concentrated sera cannot be tested safely due to the non-specific artefacts that tend to appear, this is probably true. It is also clear, however, that at least a substantial part of the 'anti-EBV negative' donors, as defined by the 1 : 10 threshold, must be negative in the biological sense, since the prospective study on infectious mononucleosis has clearly shown⁴⁴ that the disease develops only in this group, and not in persons classified as anti-EBV positive according to the same criteria.

Concerning the relationship between EBV and nasopharyngeal carcinoma, the same types of hypotheses can be discussed as for BL. The multiple virus

hypothesis would imply an NPC-specific EBV-variant. The co-factor hypothesis would lead to a consideration of both genetic and environmental factors, in view of the information on the incidence of the disease in migrant high-risk populations.⁸¹ A prospective serological study of this question would be very difficult at the present time, since NPC, unlike BL, occurs over an extremely wide age-range.

The possible significance, for the understanding of neoplastic cell behaviour, of the cell membrane changes reflected by the appearance of new antigens cannot be assessed at present, but it may be pertinent to point out that cell membrane changes are among the most significant parameters of neoplastic behaviour at present. They are almost invariably found when comparable normal and transformed cells are studied in parallel. They may concern changes in behaviour, such as contact inhibition,⁸² or altered expression of phytoagglutinin receptors^{83, 84} that may reflect a change in the synthesis of certain glycolipids,⁸⁵ and are perhaps linked to the appearance of new surface antigens.⁸⁶ Membrane-antigen changes have been demonstrated in all experimental tumors that have been thoroughly studied¹⁻⁷ and although the details concerning antigenic strength and patterns of cross-reactivity vary from system to system, they must reflect some remodelling of the membrane structure. Growth-regulating mechanisms, including both the forces that act via long-range, humoral arms and the short-range, contactual signals as well, must transmit their message to the target cell via receptors on the outer membrane. Nonlytic virus-cell interactions may result in the incorporation of virally determined (or virally derepressed) components into the membrane that render the appropriate receptors insensitive to regulation and, if this is compatible with continued cell growth and division, it may trigger neoplastic development. Since infection with potentially oncogenic viruses and the concomitant surface antigenic changes are not limited to the oncogenic target tissue but can occur in other cells as well that remain normal (i.e. subject to regulation), a tissue or cell type-specificity must be added to explain transformation. Since different tissues must obey different types of growth regulation, this is not surprising. Also, virally determined antigens may be retained while *in vivo* tumorigenic properties decrease or are lost from cell hybrid lines⁸⁷⁻⁸⁹ or from the 'revertant' forms that may arise from transformed cultures *in vitro*.⁹⁰⁻⁹² Further studies on such systems will be most interesting, not only for the understanding of neoplastic behaviour, and the possible role of membrane changes in it, but also for the understanding of normal growth-responsiveness at the cell level.

Meanwhile, the question whether EBV-associated membrane-antigens are essential for the neoplastic

behavior of BL and NPC cells is not clear. As far as NPC is concerned, such membrane-antigens have been demonstrated on derived lymphoblastoid cell lines⁷⁹ but it is not known whether they are present on the surface of the carcinoma cells. Established culture lines of BL cells carry EBV, as a rule, although at very different levels.^{29, 30} The membrane-antigen can only be demonstrated in lines with a relatively high 'EBV-load'²⁹ and is subject to environmental fluctuations.⁸³ There is at least one BL line (Raji) which contains no EBV antigen demonstrable by immunofluorescence or virus particles⁸⁴ although it carries DNA that hybridizes specifically with EBV-DNA, as already mentioned.⁸⁵ Since the Raji line can be superinfected with EB virus,⁸⁶ the absence of virus production is presumably not due to repressors. If it carries genetic information derived from EBV, it is probably a defective viral genome, lacking the cistrons that specify the membrane, capsid and early protein antigens. If there would be any assurance that the Raji line represents a neoplastic cell, this would imply that the membrane antigen is not required for neoplastic behavior. Since this question cannot be tested directly with a human cell, however, a conclusive answer is not available. Further studies on the presence of viral DNA and virus-specific mRNA in BL-derived lines, in comparison with EBV-carrying blastoid cell lines of other origin may prove very informative. In this connection, it is interesting that IM-derived lines are reportedly more prone to lose their EBV than BL-derived lines.⁸³ Thus, whereas EBV is clearly helpful in inducing lymphoblastoid transformation and facilitates the establishment of stationary suspension cultures,^{33, 97-99} there is no doubt that blastoid cell lines *can* exist without the expression of a productive EBV infection.

Turning now to the *therapeutic problems*, it seems clearly established that the host immune-response plays an important part in Burkitt's lymphoma. This is indicated by the documented occurrence of spontaneous regression,⁶² by the substantial fraction of long-term survivors, sometimes after only mild chemotherapy,^{9-11, 100} by the reactivity of the autochthonous host against its own tumor cells, indicated by the presence of humoral antibodies reacting with the surface of viable cells,^{101, 102} by the positive C1-a fixation test,¹⁰³ and by the transformation of host lymphocytes when confronted with mitomycin-treated autochthonous lymphoma cells in the mixed lymphocyte-target cell interaction test.¹⁰⁴ In addition, the processive accumulation of an IgG coating on the cell surface of tumors that persist in spite of therapy^{15, 16} together with the tetraploid (immuno-resistant?) constitution of tumors that have recurred after long-term regression²² suggests that the dynamics of immunoselection may also apply to this

human system as they do for experimental tumors.⁶¹ Immuno-resistance may be as important as drug resistance, if not more so, in frustrating therapy.

The host response to an autochthonous tumor is no less complex than other immune responses against viable cells. Different effector components interact in ways that rejection, or its opposite, enhancement, will dominate the eventual outcome. Humoral antibodies are cytotoxic in some situations whereas in others they lack demonstrable growth inhibitory effects but nevertheless manage to attach and thereby prevent the access of host lymphoid cells.^{3, 105} Recent evidence indicates that such 'blocking antibody' may play an important role in counteracting the cell-mediated host response in experimental¹⁰⁶ and human¹⁰⁷ tumors as well.

The main therapeutic dilemma is what the proper stimuli are, specific or non-specific, and how they are best administered to the immune system in order to achieve the objective, rejection, and avoid its opposite, enhancement. The rationale of introducing immune stimuli at a time when the tumor load confronting the host is minimal, i.e. after regression has been induced by chemotherapy, is obvious.^{108, 109} but the optimal form of stimulus and the best mode and timing of its administration is not. No *a priori* guidance can be given from experimental studies, because the same mode of administration, dosage, vehicle, etc, of the same preparation may favour rejection in one system and enhancement in another, and the differences depending on host species, tumor type and individual characteristics of the tumor line are immense. Ideally, it would be desirable to develop methods that allow the quantitative assessment of cell-bound immunity and the synergistic or antagonistic action of humoral antibodies in relation to it, in each untreated patient and follow it subsequently during treatment. While this should be feasible, at least in principle, its practical application is still in the future. Meanwhile, an empirical approach, based on as much rational reasoning as the experimental models will allow, may yield important information, as the work of Mathé and his group clearly indicates.¹⁰⁹

Obviously, the *prevention* approach will have to await the further clarification of the relationship between serum conversion and tumor development, preferably from a prospective study. A discussion of this beyond the general statement that the ultimate goal of an immunological approach must be prevention rather than therapy appears premature at the present time.

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