# RELATION BETWEEN EPSTEIN-BARR VIRAL AND CELL MEMBRANE IMMUNOFLUORESCENCE IN BURKITT TUMOR CELLS*

## IV. Differentiation between Antibodies Responsible for Membrane and Viral Immunofluorescence

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Previous reports (1, 2, 3) have suggested that the Epstein-Barr virus (EBV) induces the appearance of antigens on the surface of cells cultured from Burkitt's lymphomas (BL) or peripheral leukocytes from patients in the acute stage of infectious mononucleosis (IM). Various observations indicated that the cell membrane antigens are distinct from EB virion antigens. Most notable is the fact that sera were found which reacted predominantly with the cell surfaces, and little or not at all, with EBV antigens. This type of reactivity was found thus far only among sera from relatives of Burkitt tumor patients. The converse "discrepancy", high anti-EBV titers, but insignificant interaction with cell membrane antigens, was also noted in a few sera. The present report is concerned with an analysis of such differential serological activities.

### *Materials and Methods*

*Cells.*—The establishment and maintenance of cell cultures derived from Burkitt tumor biopsies and peripheral leukocytes of IM patients have been previously described (4, 5).

*Immunofluorescence Tests.*—The techniques for detection of antibodies to cell membrane

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antigens and for the titration of anti-EBV reactivity have been described (6–9).[1] The results of indirect membrane fluorescence, using a fluorescein-conjugated goat anti-human gamma chain reagent (Hyland Laboratories, Los Angeles, Calif.), are expressed in terms of fluorescence indices (FI) which were calculated by the established formula (1). Blocking tests were performed, using reagents prepared for direct membrane fluorescence by a previously described conjugation procedure.[1] The results of these experiments are expressed as blocking indices (BI) and are calculated as previously reported (3).[1] Membrane antibody titers were determined by indirect fluorescence. The serum dilution giving a FI of 0.3 was taken as the titer end point.

*Serum Absorptions.*—1 ml of Mutua serum (3) diluted 1:40 was absorbed 5 × with a total of $7.5 \times 10^7$ cultured BL cells. Absorption was done at 37°C for 1 hr, followed by overnight at 4°C.

## RESULTS

*Indirect Immunofluorescence Tests.*—Sera from nine relatives of Burkitt tumor patients (eight siblings, one father) revealed low anti-EBV levels ($\leqq$1:10), yet gave significant indirect membrane immunofluorescence (MIF) reactions, both when BL and IM cells were used as targets. In Fig. 1 the titers obtained in the MIF test with eight of these discrepancy sera are plotted against the anti-EBV titers. Also included in the figure, for comparison, are corresponding data obtained with sera from patients with BL, IM, and cancer of the postnasal space (Ca PNS). Whereas the MIF titers of sera from the three types of patients showed a good correlation with their anti-EBV levels, the sera from BL relatives clearly did not fall into this pattern.

Fig. 2 compares the MIF reactivity of the undiluted BL relative sera with the activity of the undiluted standard BL patient Mutua serum. The percentage of cells reacting with Mutua serum has been plotted against the percentage of cells which react with the various BL relative sera, tested at the same time against the same target cells by the indirect MIF test, using a goat anti-human heavy chain reagent. The spectrum of target cells used in those tests were derived from membrane-reactive or nonreactive BL and IM cell lines. As described previously (1), cells of these various lines do or do not react with Mutua serum, depending on the extent of the EBV carrier state, as well as periodic fluctuations in the persistent infection. The reasons for the fluctuations are unknown but appear to depend on the age and condition of the cultures. It can be seen that there was a direct relationship between the reactivity of the various cells with the Mutua serum and their reactivity with the various discrepancy sera tested, although the percentages of cells stained by the latter were often somewhat lower than those obtained with Mutua serum. This indicated that although essentially anti-EBV-negative, the various BL relative sera contained antibodies capable of reacting with the membranes of BL and IM cells in a way similar to the reactivity of the Mutua serum.

[1] Goldstein, G., G. Klein, G. Pearson, and P. Clifford, 1968. Direct membrane immunofluorescence reaction of Burkitt lymphoma cells in culture. *Cancer Res.* In press.

*Blocking Tests Against F-Mutua (Fluorescein-Conjugated Globulin of Mutua's Serum).*—To investigate further the specificities of the described reactions, sera from relatives of BL patients were tested for blocking activity against F-Mutua. The BI values plotted against anti-EBV titers are shown in Fig. 3, chart A. For comparison, the BI values of the BL, IM, and Ca PNS sera used in Fig. 1

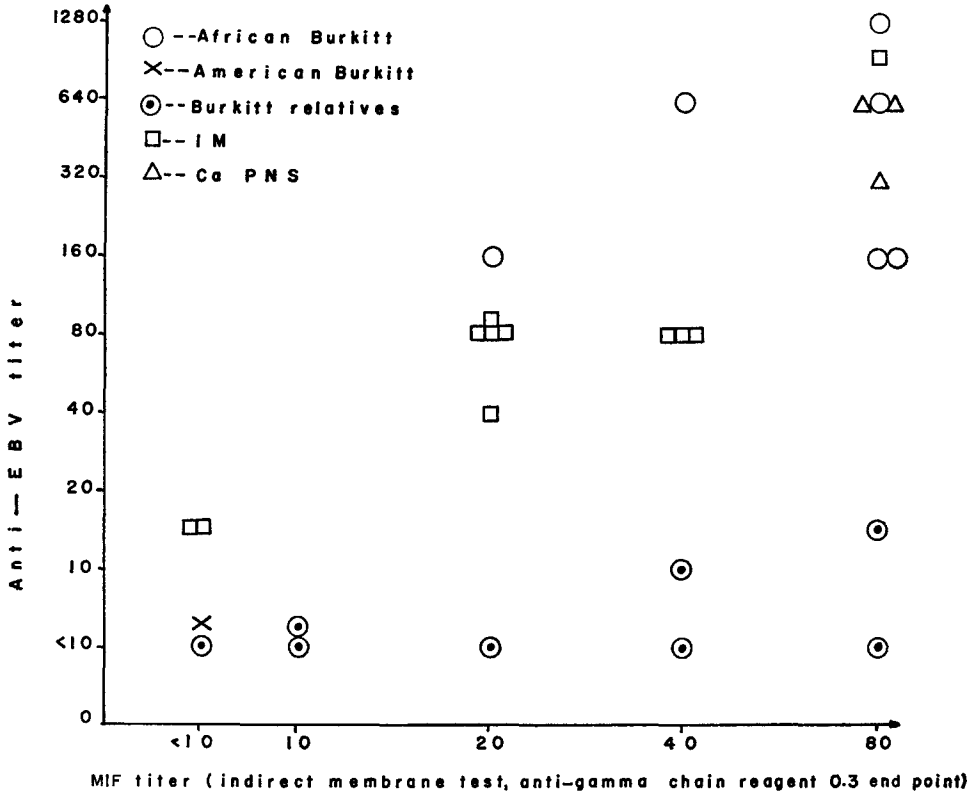


FIG. 1. Indirect membrane immunofluorescence and anti-EBV titers of sera from relatives of BL patients as compared to some BL, IM, and Ca PNS reference sera.

are also plotted against their anti-EBV titers to illustrate the usual relationship between BI and anti-EBV values. As mentioned earlier, the BL relative sera were negative or low with regard to their level of antibodies to EBV, with one notable exception (not included in the previous test), which revealed an anti-EBV titer as high as found in most Burkitt patients' sera (3). This serum also showed a very high blocking activity, whereas the sera with no or low anti-EBV activity in this category showed only relatively low (BI of 0.2–0.5) or insignificant blocking. Two sera from other categories also seemed to fall outside

the usual pattern. These had high anti-EBV titers but low blocking activity and came from patients with Ca PNS (10). The possible significance of these sera will be discussed later.

The weak blocking of F-Mutua by BL relative sera as compared to the strong reactivity of sera from patients with BL and certain cases of Ca PNS raised the question whether qualitative or quantitative differences in antibodies

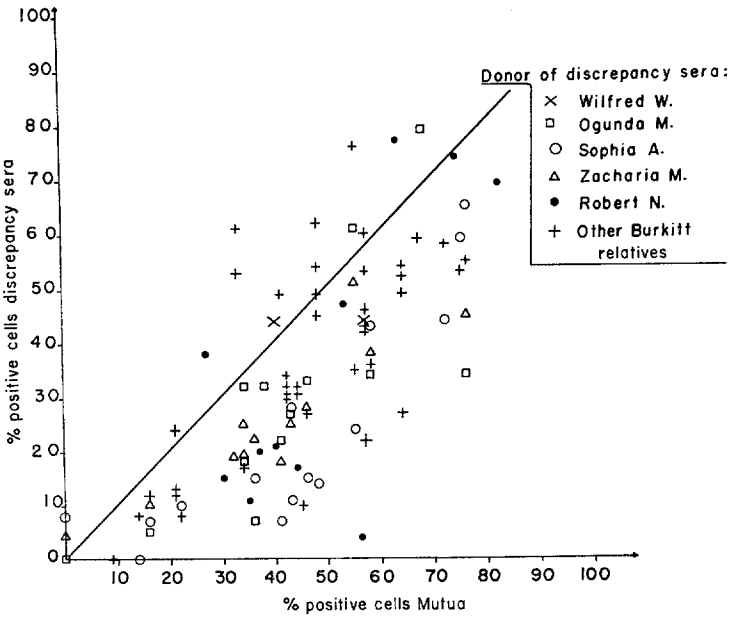


FIG. 2. Indirect membrane immunofluorescence reactivity of EBV-negative, membrane-positive BL patients' relative sera compared with the reactivity of Mutua serum against the same spectrum of reactive and nonreactive BL and IM cells. Goat anti-human gamma chain reagent was used in these tests.

were responsible for the differential behavior of the sera. This question was explored by plotting the MIF titers of the various sera against their blocking indices (Fig. 3, chart B). It appeared that the MIF titers of sera, other than those from relatives of BL patients, with two exceptions, were directly related to their BI values, since the mean titers seemed to fall into a narrow band. The sera of the relatives also conformed to some extent to this pattern, although their BI values generally fell into the lower ranges for given MIF titers, and in two cases were well below the ranges observed. The four sera which clearly fell outside this pattern all had MIF titers of $\geqq 1{:}80$, yet failed to block F-Mutua staining in a significant manner. The two sera from BL relatives had anti-EBV titers of $\leqq 1{:}10$. The other two sera, derived from patients with Ca PNS, had

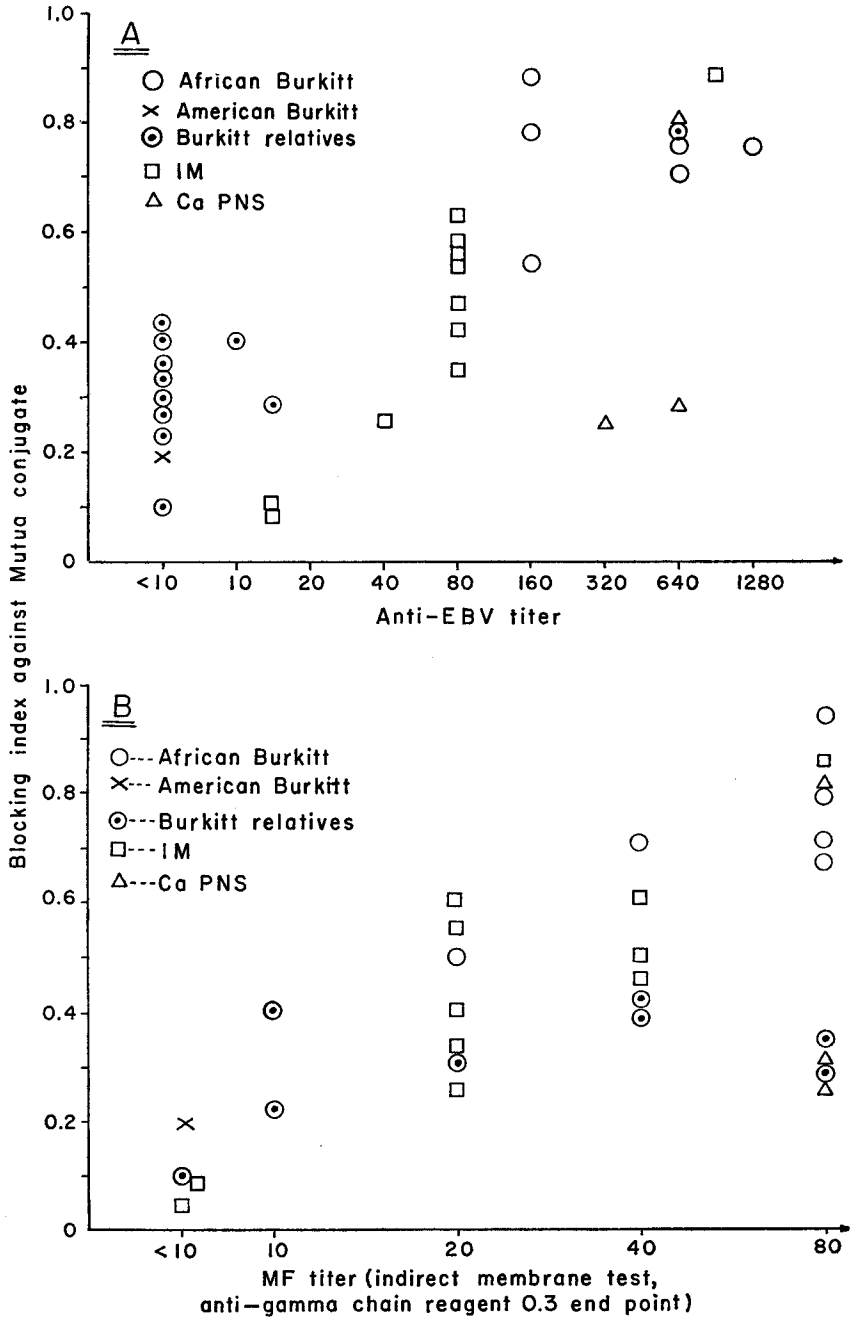


FIG. 3. Relationship between the blocking activity (BI), against Mutua conjugate, of representative sera of the various categories indicated, and (A) anti-EBV titer, and (B) indirect membrane immunofluorescence titer.

anti-EBV titers of 1:320 and 1:640, respectively, and were in fact the same sera which failed to show the usual correspondence between anti-EBV and BI reactivities (Fig. 3, chart A). These results revealed that MIF and BI reactivities do not necessarily parallel each other.

*Blocking Tests with F-Robert.*—The above observations indicated the need of FITC conjugates prepared from sera of BL relatives for blocking tests. Correspondingly, the gamma globulin of an 18 year old relative, Robert N. (brother

TABLE I

*Summary of Blocking Tests with BL Relative Sera against F-Mutua and F-Robert Compared with Anti-EBV and MIF Titers of Same Sera*

| Name | Status | Titers | | BI | |
|---|---|---|---|---|---|
| | | Anti-EBV | MIF* | F-Mutua | F-Robert |
| Mutua N. | Burkitt's lymphoma | 160 | >80 | 0.78 | 0.90 |
| Robert N. | BL relative | <10‡ | | | |
| | | 40 | ≥80 | 0.26 | 0.91 |
| Beth N. P. | BL relative | <10 | 20 | 0.35 | 0.75 |
| Ogunda M. | BL relative | <10 | 40 | 0.35 | 0.73 |
| Salim M. | BL relative | <10 | 10 | 0.30 | 0.94 |
| Sophia A. | BL relative | <10 | ≥80 | 0.31 | 0.54 |
| Zacharia M. | BL relative | <10 | <10 | 0.14 | 0.15 |
| Wanambisi | BL relative | 10 | 40 | 0.40 | 0.73 |
| Berit | Control (Swedish) | 20 | N.T.§ | 0.16 | 0.17 |
| Lameck W. | Embryonal sarcoma (African) | <10 | N.T. | 0.05 | 0.00 |
| Proud | Isoantiserum (French) | 5–40 | N.T. | 0.10 | 0.00 |

* FI, 0.3 as titer end point.

‡ Anti-EBV values from two separate serum samples.

§ Not tested.

of Burkitt patient Fanis A., Kenya Cancer Council [K.C.C.] No. 300), was conjugated with FITC and used for direct membrane fluorescence and for blocking tests. When unconjugated and used in the indirect fluorescence test, this serum showed, with a similarity to the membrane-positive BL relative sera, a reactivity against different cell lines that closely paralleled the reactivity of the same cells with Mutua's serum in the indirect test, as shown in Fig. 2. This serum was also tested for isoantibodies by leukocytotoxicity against 37 cell donors. The results were negative.[2] When the stainability of different target cells with the fluorescein-conjugated globulin of Robert N.'s serum, F-Robert, was compared with the staining of the same cells with F-Mutua, a generally

[2] Terasaki, P. I. Personal communication.

good correlation was obtained. This finding is in agreement with the results of the indirect test (Fig. 2). Thus the antibodies present in Robert's serum are directed against antigen(s) present on BL and IM cells, the expression of which is also demonstrated by Mutua's serum.

Blocking tests, using F-Robert and various types of sera, revealed that six sera from BL and three sera from Ca PNS patients tested were strongly posi-

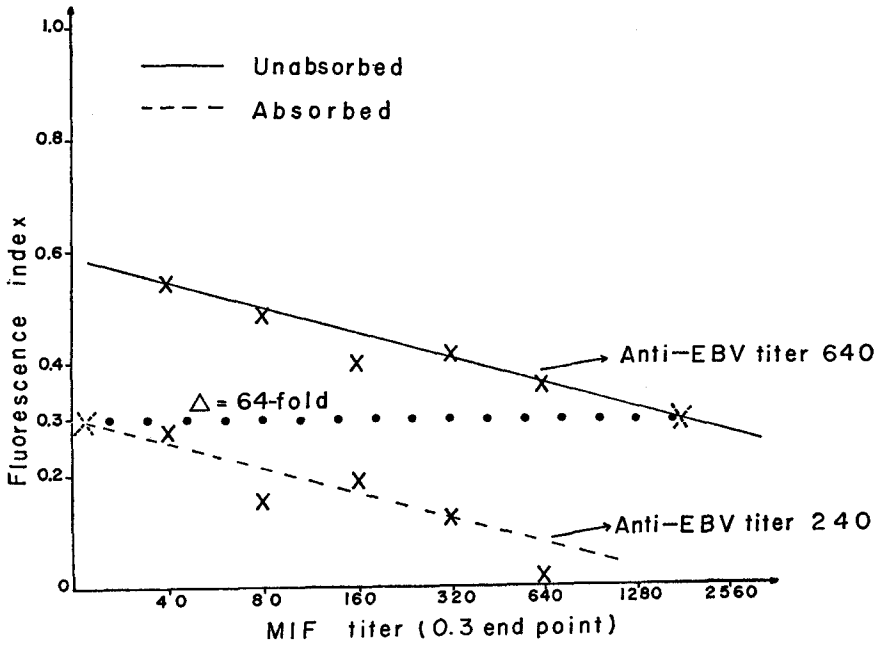


FIG. 4. Comparison of anti-EBV and MIF titers of Mutua serum before and after absorption with membrane-reactive BL cells. A fluorescence index (FI) of 0.3 was taken as the end point for determining the indirect membrane immunofluorescence (MIF) titer.

tive, whereas among 11 control sera from African patients, with or without neoplastic disease, eight were negative and three showed limited extents of blocking. It is significant that six of seven BL relative sera examined strongly blocked F-Robert (Table I). Of special interest is the fact that no blocking was obtained with an isoantiserum from a multiply transfused French patient (Proud) which was previously found to react with all target cells in the indirect MIF test (7). In contrast to the above results with F-Robert, the BL relative sera blocked staining by F-Mutua only to minor or insignificant extents. These results indicate that the serum of Robert shares antibodies with the serum of Mutua, but that Mutua's serum contains additional antibodies which are either absent in Robert's serum or present at insufficient levels to compete

with Mutua's serum, since F-Mutua is not blocked by Robert's serum. The antibody present in Robert's serum appears to be present also in sera of other relatives of BL patients.

*Demonstration of Sera with High Anti-EBV and Low Blocking Activity Against Conjugated Mutua Serum.*—As shown earlier in this paper, as well as in the preceding paper of this series (3), several sera were observed with high anti-EBV activity but low ability to block the direct membrane staining by the Mutua conjugate. It was conceivable that these sera, some of which were positive in indirect MIF tests, may contain part but not all of the antibody spectrum present in Mutua's serum, since they did not block F-Mutua. Alternatively, their antibody activity may be absent from the Mutua conjugate. It is of interest that two of the sera which were also tested for blocking activity against F-Robert showed higher BI activity against this reagent than against F-Mutua. Since exceptional sera may permit further dissection of the serological interactions, such sera are being searched for and studied in more detail in cross-blocking experiments which are presently in progress.

*Serum Absorption Experiments.*—To provide further evidence that the antibodies involved in the anti-EBV and membrane immunofluorescent reactions are distinct, Mutua serum was absorbed with cultured Burkitt tumor cells that had been pretested with F-Mutua for MIF antigens. The absorbed serum was then titrated for antibodies to both EBV and membrane antigens. The results of this experiment are shown in Fig. 4. If the dilutions of the absorbed and unabsorbed serum which gave MIF indices of 0.3 were compared, it was seen that the MIF titer of the absorbed serum had decreased approximately 64-fold, whereas the anti-EBV titer had declined only about 3-fold.

## DISCUSSION

Recent results (3) have established that sera from most patients with Burkitt's lymphoma, infectious mononucleosis, and certain patients with nasopharyngeal cancer contain antibodies directed against the membranes of cells from EBV-carrying cultures, as well as against EBV itself. However, discrepancies have been noted which have suggested that two different types of antigens and antibodies are involved in the two tests. The results of experiments reported in this paper tend to provide further support for this suggestion.

The most convincing evidence was provided by absorption of the Mutua serum with membrane-antigen–positive BL cells. Membrane activity was removed without significantly affecting the anti-EBV titer of the serum.

Further evidence was provided when donors of sera containing high anti-membrane but low anti-EBV activities were found among relatives of Burkitt patients. The antibody specificities of these sera were studied extensively by both indirect and blocking of direct membrane immunofluorescence techniques. The results suggest that the antibodies found in the serum of Mutua, a BL

patient, share at least some specificities with antibodies found in these sera. This was established by demonstrating a correspondence of stainable cells in indirect and direct membrane immunofluorescence tests on the various target cells and in blocking experiments against F-Mutua and a conjugated BL

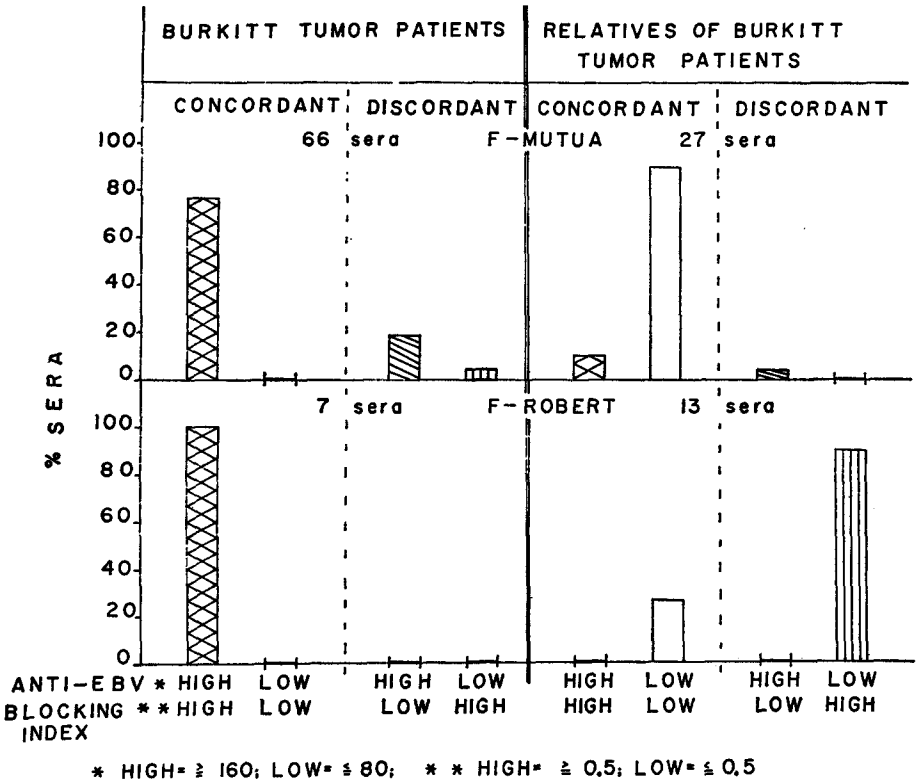


FIG. 5. Blocking patterns established for sera from BL patients and relatives of BL patients against F-Mutua and F-Robert. The number of such sera in the different categories is shown in chart. Other symbols are indicated on the chart.

relative serum, F-Robert. These sera did not block the F-Mutua, or blocked it only to insignificant or partial extent. F-Robert, on the other hand, was blocked by the sera of BL relatives and by Mutua, but not by various control sera which included an isoantiserum, thereby establishing a partial identity among the blocking sera. These blocking patterns established for sera from BL patients and BL relatives against F-Mutua and F-Robert are presented in Fig. 5.

It is interesting to note that although Robert serum did not block F-Mutua, Mutua serum did block F-Robert. Since Robert had a MIF titer of $\geq$1:80, the

failure of Robert to block Mutua would not appear to be explained by quantitative but more likely by qualitative factors. One possible explanation for this paradox is that Mutua serum contained anti-membrane antibodies of more than one specificity. If this were true, and Robert lacked one of these specificities, one would not expect Robert to block F-Mutua, but Mutua should block F-Robert. This might also be the explanation for the failure of some strongly EBV- and MIF-positive sera to block F-Mutua. This interpretation obviously requires further substantiation.

Sera with positive MIF but low or no anti-EBV activities might denote: (*a*) that in rare instances the viral genome persists in some cells without replication of infectious virus but induces surface antigens in some cells or (*b*) that an antigenically distinct herpes group virus induces similar antigens. It would be difficult to see how interpretation (*b*) could account for the involvement of relatives of BL patients in this type of discrepancy. Interpretation (*a*) could denote that the virus persists in certain healthy individuals in a "nonvegetative" form which is nevertheless capable of inducing surface antigens. This might happen, perhaps particularly, in direct patient contacts, although the significance of patient contact for this type of serum reactivity remains to be investigated further, using carefully matched controls.

The opposite discrepancy, sera with high anti-EBV levels that fail to block the Mutua conjugate might indicate: (*a*) that the presence in an individual of numerous cells with surface antigens absorbs the corresponding antibody from the serum and body fluids. This situation might occur in patients with carcinoma of the postnasal space, provided that the cells of this tumor also contain the surface antigen, just as does the surface of Burkitt lymphoma cells. The same might hold occasionally for patients with large, rapidly growing recurrent Burkitt's lymphomas (3); or (*b*) that too few cells with surface antigen are present to keep the corresponding antibodies at levels sufficient for their detection. This might occur in infectious mononucleosis where the bulk of the antigen-containing cells may disappear after temporary proliferation. Anti-EBV titers are less subject to fluctuations and persist for indefinite periods of time. There is evidence that EBV (little as may be there) persists not only in the tumors, but also in other cells of the lymphatic system. One may assume then that EBV is possibly more antigenic than the surface antigen(s), or alternatively, that it is largely hidden inside the cells and less available for absorption of circulating antibodies.

Whatever the ultimate explanation, it seems clear that the MIF and EBV antigens are distinct, as are the corresponding antibodies. Furthermore, the differential blocking of F-Mutua and F-Robert by various sera suggests the existence of at least two membrane antigens. The question whether the surface antigen(s) have any relation to malignant transformation, that is, whether the EBV-related surface antigen(s) result from the action of an agent capable of

such transformation, or represents merely a passenger, cannot be answered at present.


## SUMMARY

Previous reports (1, 2) have established that the expression of certain distinctive membrane antigen(s) on the surface of Burkitt's lymphoma (BL) and infectious mononucleosis (IM) cells is dependent on the presence of Epstein-Barr virus (EBV) in the cell line. The investigations reported here provide evidence that antibodies directed against EBV antigens, as revealed by the immunofluorescence test on acetone-fixed smears (8), and the membrane reactive antibodies, although often present in the same serum, are nevertheless distinctly different. Absorption of Mutua serum, the standard reference serum for demonstrating membrane antigen(s) on BL and IM cells, with BL cells completely removed anti-membrane activity without significantly affecting the anti-EBV antibody titer. Furthermore, sera were found which contained one type of antibody but not the other. Sera with high anti-membrane but low anti-EBV activity were found among relatives of BL patients. These sera reacted with the membranes of EBV-carrying BL and IM cells in essentially the same way, i.e., against the same spectrum of target cells, as the EBV-positive Mutua serum. They were unable to block the membrane reactivity of FITC-conjugated Mutua serum, however. In some cases they showed weak but incomplete blocking. One such EBV-negative, membrane-positive BL relative serum (Robert) was conjugated with FITC and used for direct staining of BL and IM cells. Again, this conjugate reacted against the same target cell spectrum as a Mutua conjugate, but its reactivity was completely blocked by a number of Burkitt patients' sera, although unconjugated Robert serum did not block the Mutua-conjugate. A number of other membrane-positive BL relative sera also failed to block Mutua, but completely blocked the Robert conjugate. A number of Swedish and African control sera and an isoantiserum gave no blocking against Robert conjugate. It therefore appears that the Mutua conjugate contains at least two antibody specificities against the EBV-determined membrane antigens. One, but not the other, is shared with the antibody specificity present in Robert's serum and a number of other sera from relatives of BL patients.


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