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

III. Comparison of Blocking of Direct Membrane Immunofluorescence and Anti-EBV Reactivities of Different Sera

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In the first paper of this series (1) it was shown that the membrane antigens of cultured Burkitt lymphoma (BL) cells (2, 3) depend not only upon the presence of Epstein-Barr virus (EBV) (4), but also upon the extent of the persistent EBV infection. The membrane antigens were demonstrated by indirect immunofluorescence tests with live cells and the standard serum obtained from Mutua N., a Burkitt tumor patient, who possessed no detectable isoantibodies (3). The percentage of cells with EBV antigen was determined by direct immunofluorescence tests with acetone-fixed cell smears and fluorescein isothiocyanate (FITC)-conjugated pooled human gamma globulins (5). In the second paper of this series (6) it was shown that cultured blastoid cells of EBV-positive lines derived from leukocytes of patients in the acute stage of infectious mononucleosis (7) may possess membrane antigens which are similar to those detected in BL cells; and that in the course of infectious mononucleosis (IM) patients develop antibodies which react with membrane antigens of both BL and IM cells. In both studies observations were made which suggested that the

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antigens involved in the two types of immunofluorescence tests are distinct. Cells showing membrane immunofluorescence (MIF) generally exceed those containing EBV antigens by a factor of more than 10. There was no constant ratio between MIF-positive and EBV-positive cells, and cells from biopsies or recently initiated cultures of Burkitt tumors often revealed the presence of membrane antigens, while none, as a rule, contained detectable EBV antigens. Finally, antibodies to membrane antigens were found to develop in the course of IM at an apparently slower rate than those to EBV antigens.

The indirect MIF test has been replaced recently by blocking of direct membrane immunofluorescence. It was shown[1] that conjugation of the gamma globulin fraction of the standard Mutua serum with FITC yielded a reagent suitable for direct staining of membrane antigens. The direct staining of cells with this reagent correlated well with results obtained by the indirect technique, in that both procedures stained similar percentages of cells in positive lines and gave no reactions with cells of negative lines. The direct staining technique offers the prospect of a more refined tool for a critical comparison of antibody specificities in various sera than the indirect method, in that it permits blocking tests and thereby restricts the assay to the spectrum of antibodies represented in the conjugate. Preliminary blocking data demonstrated that direct MIF was inhibited by unlabeled sera from BL patients, but not by a number of control sera, including three isoantisera from multiply transfused patients.[1] This technique has been applied now to numerous additional sera from various types of donors with known levels of antibodies to EBV. The results to be reported here provide further evidence for a relationship between MIF and EBV, as well as for the fact that the tests involve different antigens.

Materials and Methods

Cells.—The establishment and maintenance of the cell lines used in these investigations from Burkitt tumor biopsies and peripheral leukocytes of IM patients have been described elsewhere (7, 8).

Sera.—The sera used in this study were obtained from patients with different malignant and nonmalignant diseases as described in the text. Controls included sera derived from healthy African and non-African donors and from four multiply transfused patients.

Anti-EBV Immunofluorescence Test.—The procedures for staining acetone-fixed tissue culture cells by direct and indirect immunofluorescence and for the titration of antibodies to EBV have been fully described (5, 9).

Direct Membrane Immunofluorescence.—The preparation of the direct membrane staining reagent, F-Mutua (gamma globulin fraction of standard Mutua serum conjugated with FITC), from an antiserum obtained from African Burkitt's lymphoma patient Mutua N. (Kenya Cancer Council [K.C.C.] No. 454), which is used as the standard reference serum to demonstrate the presence of distinctive membrane antigen(s) on BL and IM cells (1, 6), has been described in detail.[1] The procedure for direct membrane staining on living BL and IM

[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.

cells has also been described.[1] In blocking tests, the cells were first incubated with unlabeled serum. After washing, the cells were then incubated with F-Mutua and processed in the usual manner.[1] The results of blocking tests are expressed as blocking indices (BI). The latter are calculated by subtracting the per cent positive cells after incubation with unlabeled serum followed by the conjugate, from the per cent positives with conjugate alone without unlabeled erum, divided by the latter.[1]

RESULTS

Sera from patients with Burkitt's lymphomas (BL), cancer of the postnasal space (Ca PNS), and infectious mononucleosis (IM), as well as various control sera, were tested for antibodies capable of inhibiting the direct membrane staining of BL cells by F-Mutua. The blocking indices (BI) of these sera were plotted against their anti-EBV titers. Three or more blocking tests were performed with each serum and the average BI calculated. The results of all sera tested for both reactivities are shown in Fig. 1. Different symbols are employed for sera from various groups of individuals. In general, sera with high anti-EBV titers yielded high BI values (40 of the sera studied gave anti-EBV titers $\geqq 160$ and BI >0.5), while sera that were negative or low with regard to anti-EBV activity failed to block F-Mutua to any significant extent (55 of the sera studied gave anti-EBV titers $\leqq 80$ and BI <0.5). Several exceptions were noted, however, in that seven sera showed low anti-EBV reactions ($\leqq 80$) but a high BI (>0.5), and another eight sera revealed high anti-EBV titers ($\geqq 160$) and a low BI (<0.5). These discordant sera, providing evidence that different antibodies are involved in the two reactions, have been subjected to further intensive study as will be reported separately (10).

The various categories of sera were analyzed separately as shown in Fig. 2. It can be seen that 18 of 20 African Burkitt patients' sera tested (Fig. 2, chart A) had both high anti-EBV and high blocking activity. The two discordant sera with high anti-EBV titers ($\geqq 160$), but low BI values (<0.5) came from a single patient, and were taken either shortly prior to recurrence of the tumor after a long-term regression, or later, after intensive chemotherapy. If the total number of sera tested are considered, including those which were tested only once or twice in the blocking test, 48 of 61 African Burkitt patients' sera had both high anti-EBV and high blocking activity. Three of six non-African Burkitt patients' sera tested had anti-EBV and blocking reactivities either both high or both low.

The BI values of IM sera (Fig. 2, chart B) also seem to correlate well with anti-EBV titers. The negative serum was a pre-IM serum and those with low BI values were early acute stage sera (11). In general, it appears that anti-EBV titers, as well as BI values attained in IM, are somewhat lower than in the cases of BL.

Sera from patients with Ca PNS (12) seem to fall into two major groups (Fig. 2, chart C): (*a*) sera with high anti-EBV and high blocking levels (19

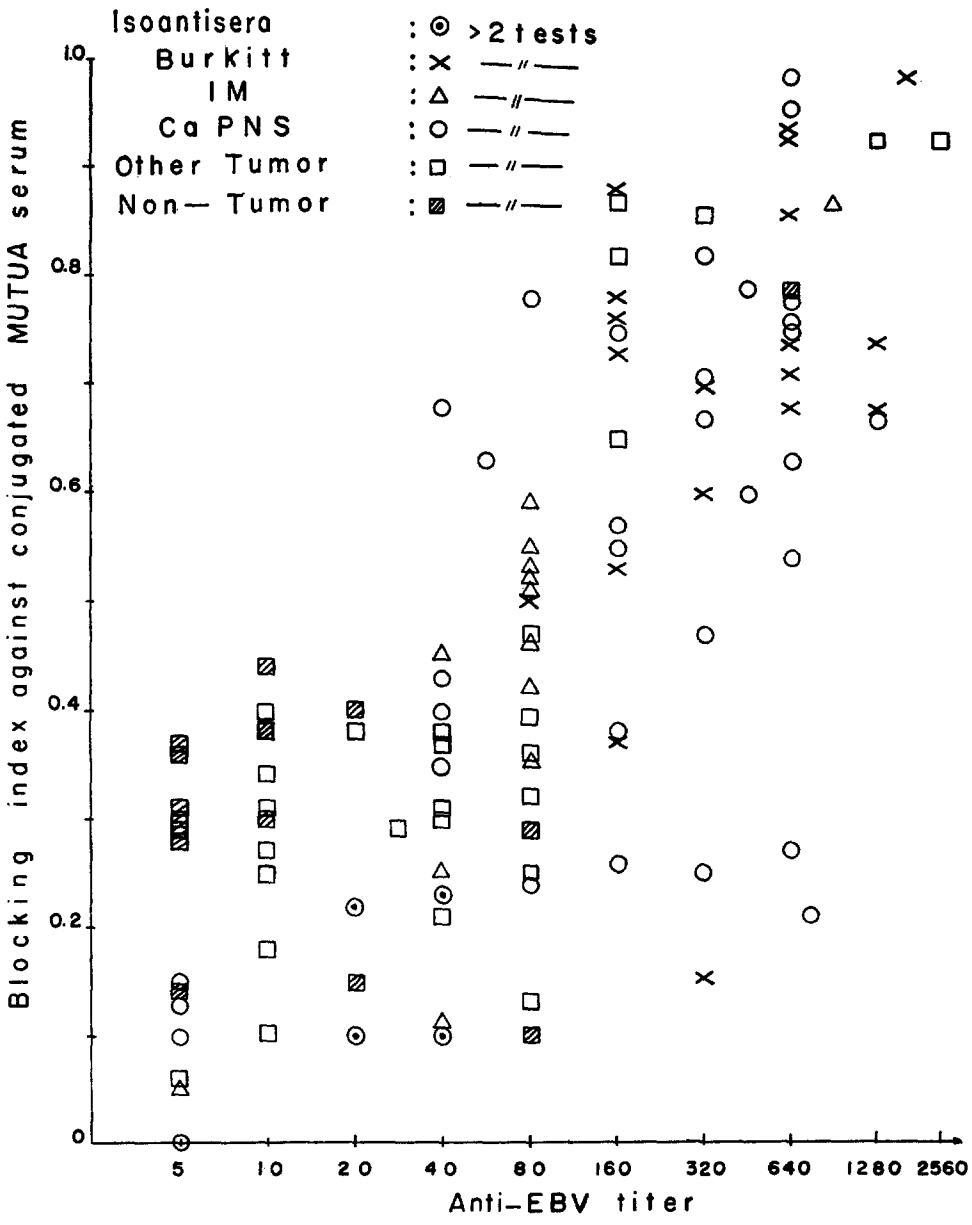

FIG. 1. Relationship between anti-EBV titer and blocking index (BI) against FITC-conjugated Mutua serum in sera from patients with different neoplastic and nonneoplastic diseases and from normal controls, including four broad-spectrum isoantisera from multiply transfused donors. Three or more blocking tests were performed with each sera and the average BI calculated.

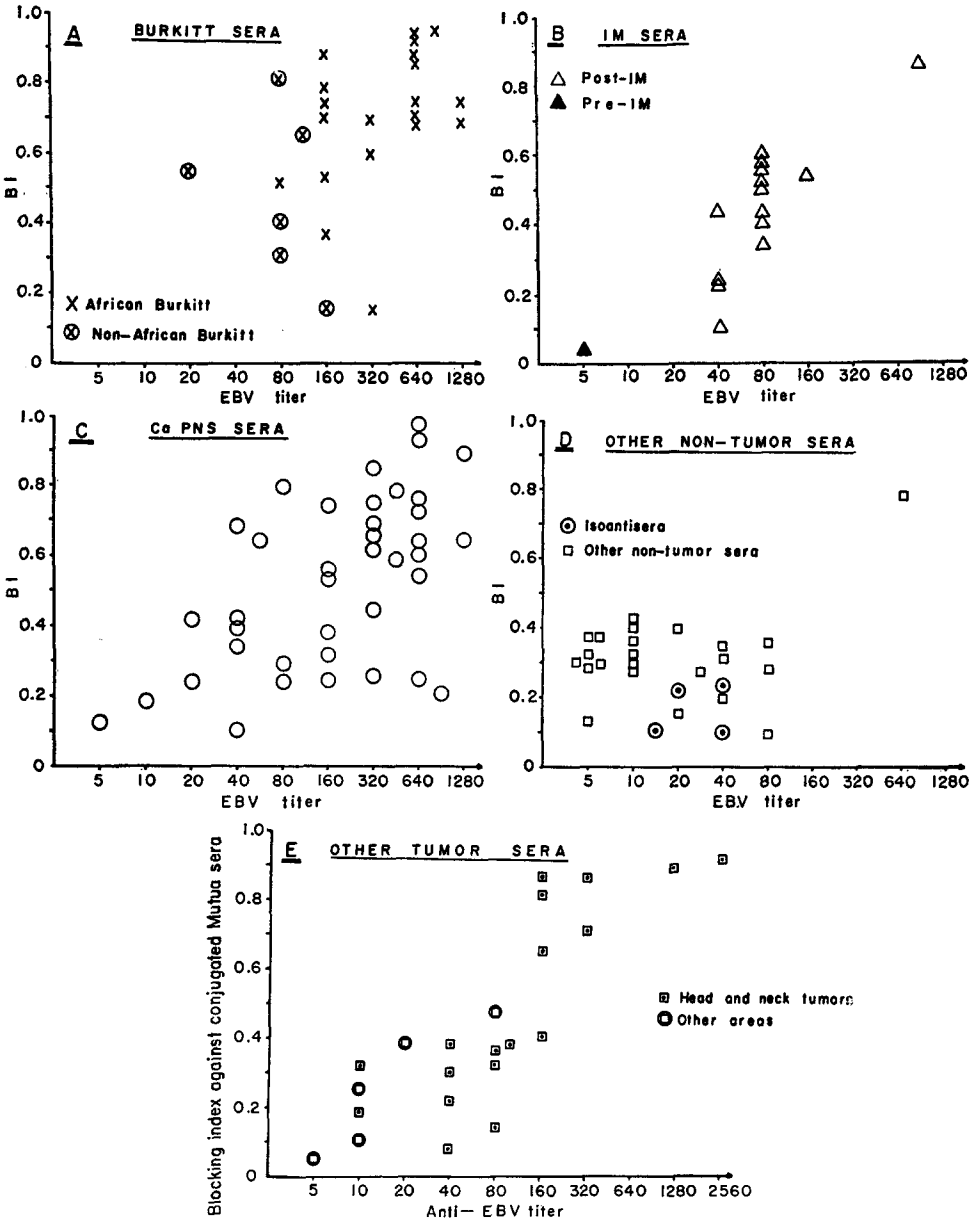

FIG. 2. Relationship between serum anti-EBV and BI values in different disease categories, as indicated.

sera); and (*b*) sera with low anti-EBV and low BI values (10 sera). Again, there seems to be a correlation between anti-EBV titer and BI, but some exceptions were noted. Three sera gave low anti-EBV and high blocking values, and seven sera gave high anti-EBV titers and low BI values.

The results obtained with control sera, including four different isoantisera, and sera from patients with nontumor diseases are shown in Fig. 2, chart D. It is especially noteworthy that the isoantisera gave low BI values, correlating with their low anti-EBV titers, even though they reacted strongly with the membranes of BL and IM cells, as shown by indirect immunofluorescence tests (3). This group also contained sera from relatives of Burkitt's lymphoma patients. These sera have been of particular importance in providing evidence that two antibodies are involved in the different reactions (10).

Sera from patients with other types of tumors are shown in Fig. 2, chart E. Different symbols have been used to distinguish sera from patients with head or neck tumors from sera from patients with tumors in other regions. It can be seen that sera from the first group tended to react either strongly or weakly in both tests, similar to the observations made with sera from Ca PNS patients. The second category of sera gave mainly low anti-EBV and low BI values.

DISCUSSION

Antibodies reacting with living Burkitt tumor or IM cell membranes (2, 3, 6) and with acetone-fixed cell smears (5, 7, 9) have been detected with the use of indirect fluorescent techniques. These investigations have provided evidence that EBV is related, in some way, to the expression of membrane antigen(s) on BL or IM cells. In such indirect systems, however, it is not feasible to compare the specificities of antibodies in different sera. It is conceivable, therefore, that antibodies detected in different sera are reacting with different antigen(s) expressed in or on the same cell. The use of direct membrane immunofluorescence, however, eliminates this problem by allowing for blocking tests which will only detect antigenic sites common to the blocking and labeled sera. Furthermore, recent evidence (10) suggests that blocking is a "titer-related" function which permits a quantitative, as well as qualitative, comparison between sera.

The conjugate used for the blocking tests described above was prepared from serum of an African Burkitt's lymphoma patient, Mutua N.[1] His serum has previously been shown to be free of detectable isoantibodies (3) and has been used as the standard reference serum to demonstrate the presence of distinctive membrane antigen(s) on BL and IM cells (1, 6).

Comparison of anti-EBV titers with the blocking indices (BI) of sera from patients with BL, IM, Ca PNS, as well as from various control groups, permits a number of conclusions or suggestions. In general, it was found that sera with high anti-EBV titers gave high BI values and vice versa, but a number of exceptions were noted. These involved several groups of patients.

The majority of the sera from patients with BL revealed high levels of reactivity in both tests. Low BI values but high anti-EBV were noted in several sera from one patient, which might be referable to different stages of the disease, and to treatment by chemotherapy. Further study is needed to clarify this point. It is conceivable that membrane-reactive antibodies are removed from the circulation during recurrence of the tumor, possibly by adsorption onto the growing tumor or by combination with antigen released from tumor cells destroyed by chemotherapy. Furthermore, antibody levels may decline as a result of the immunosuppressive action of the therapy, although this has not been noted with respect to anti-EBV antibodies.

TABLE I

Summary of Results Obtained with 279 Sera in Blocking of Membrane Immunofluorescence and Anti-EBV Titrations

Blocking Index	Anti-EBV titer	
	High (≧160)	Low (≦80)
High (≧0.5)	75 (+27) 102/279 = 37%	17 (+5)* 22/279 = 8%
Low (<0.5)	17 (+14) 31/279 = 11%	97 (+27) 124/279 = 44%

* Figures in parentheses represent the number of sera that have been tested only once in blocking tests; all other sera have been tested two or more times.

Patients with Ca PNS seem to fall into two groups: (*a*) one with high anti-EBV and high BI values; (*b*) one with low anti-EBV and low BI values. Sera from patients with other tumors of the head or neck region also seem to fall into these two groups. With regard to Ca PNS, it may be interesting to compare the histology of tumors in these two groups, since this class of malignancies is undoubtedly not uniform.

The exceptions in the correspondence of the results of the two assays provide further evidence that different antibodies are involved in the respective reactions. It is evident that, under some circumstances, one antibody is present, unaccompanied by the other. These "discrepancies" will be analyzed further in the succeeding report (10).

Sera tested for both antibodies can be divided into four different groups (Table I): those showing (*a*) high anti-EBV titers (1:160 or more) and high BI values (0.5 or more); (*b*) low anti-EBV (1:80 or less) and low BI (less than 0.5); (*c*) high anti-EBV and low BI; and (*d*) low anti-EBV and high BI. The majority of the two tests agree in that they either gave high or low values in both tests; this was the case in approximately 80% of the sera. This type of distribution would not be expected if the expression of each antigen was entirely independent

of the other. If they were independent, one would expect a random distribution. These results suggest, therefore, that the expression of both types of antigens are interrelated. It is tempting to speculate that the virus controls the production of the membrane antigen(s), as appears to be true with oncogenic DNA viruses of animals (cf. 13).

SUMMARY

Sera from patients with Burkitt's lymphoma (BL), infectious mononucleosis (IM), carcinoma of the postnasal space (Ca PNS), and various controls were investigated for antibodies against the Epstein-Barr virus (EBV) by immunofluorescence on acetone-fixed smears (5) and for antibodies against the distinctive antigenic sites expressed on the surface of viable lymphoblastoid cells within EBV-carrying culture lines (1). The latter were studied by the blocking of direct membrane staining with FITC-conjugated Mutua serum. This serum has been derived from a Burkitt's lymphoma patient in long-term regression after chemotherapy and is free from detectable isoantibodies. It has been used previously as a standard of reference to demonstrate the presence of the membrane antigen(s) on all lines derived from BL biopsies and leukocytes from IM patients. It was found that 102 of 279 (37%) of the sera tested had high anti-EBV titers ($\geqq 80$) and high membrane-blocking (BI > 0.5) activity, 124 of 279 (44%) of the sera were low in both tests, 22 of 279 (8%) had low EBV titers ($\leqq 80$), in spite of a high blocking index, and 31 of 279 (11%) of the sera were low in blocking activity (<0.5), in spite of a high EBV titer. The two tests thus gave concordant results with 81% and discordant with 19% of the sera.

The majority of sera from BL patients were high in both tests. IM sera also showed a relationship between the two antibody activities but, in general, both activities were lower than in BL cases. Ca PNS sera seemed to fall into two main groups: (*a*) high anti-EBV, high blocking or (*b*) low anti-EBV, low blocking. Control sera, including four isoantisera, showed predominantly low reactivities in both tests.

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