

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

I. DEPENDENCE OF CELL MEMBRANE IMMUNOFLUORESCENCE ON PRESENCE
OF EB VIRUS

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Two types of immunofluorescence tests are being applied to studies of Burkitt's lymphoma. In one of these (1, 2), cells from fresh biopsies or from certain lines of tumor cultures are stained vitally by the indirect technique, using sera of patients with this malignancy for the primary reaction. This test reveals surface antigens of tumor-derived cells which are not present in the membranes of fresh bone marrow cells of autochthonous or other Burkitt tumor patients. It will be referred to as membrane immunofluorescence or MIF test. In the other test (3), acetone-fixed smears of cultured Burkitt tumor cells are stained by the indirect method with various human sera or by the direct technique with fluorescein isothiocyanate-conjugated human γ -globulins. This test detects the fraction of cells in various lines which produce Epstein-Barr or EB virus particles and therefore will be referred to as EBV test. The EBV-specificity of the reactions obtained is attested by the facts that (a) there exists a good correlation between the numbers of cells in individual lines which yield immunofluorescence and cells revealing herpes group virus particles on electron microscopic examination (3-5); (b) manipulations of the cultures which increase or decrease the numbers of fluorescent cells lead to similar changes in the numbers of

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cells which harbor virus particles (4-6); (c) sera which elicit immunofluorescence contain antibodies to the EB virion as evident from antibody coating and agglutination of partially purified virus particles (7, 8); (d) radioautography 1-2 days after a ^3H -thymidine pulse has shown (9) that the majority of fluorescent cells contain isotope in the cytoplasm (viral DNA) whereas in nonfluorescent cells the label, if present, is restricted to the nucleus (cellular DNA); and (e) examination of individual fluorescent cells has revealed that they contain numerous virus particles (9, 10).

It was of interest to determine whether and to what extent the membrane immunofluorescence reaction depends upon the presence of EBV in blastoid cell populations. In the first series of experiments, attempts were made to correlate the MIF activities of various cell populations, as elicited with the aid of a standard serum, with the extents of the EBV infection. The results are presented below.

Materials and Methods

Cells.—The preparation of cell suspensions from Burkitt tumor biopsies and the establishment and maintenance of the cultures employed in these studies have been described (1, 2, 11).

Membrane Immunofluorescence Test.—The techniques employed have been reported in detail (1, 2, 12). It was known that certain sera, and particularly those derived from Burkitt patients whose tumors went into long-lasting remission after chemotherapy, gave a high incidence of positive reactions with cells from Burkitt lymphoma biopsies, but not with cells from fresh bone marrow biopsies of Burkitt patients, allogeneic lymph node cells, or allogeneic leukemic cells of various kinds (13). For this reason, one particular serum was selected as standard and used exclusively in the present studies. This serum was derived from the donor Mutua (Kenya Cancer Council [K.C.C.] No. 454), a 5 yr-old boy with histologically confirmed Burkitt lymphoma who has been in maintained remission after chemotherapy for more than 3 yr. This serum, which has been studied in great detail, lacked detectable isoantibodies, as judged by lymphocytotoxicity, leukoagglutination, and membrane immunofluorescence with bone marrow cells from donors of known leukocyte antigen groups (2). It reacted well against 13 of 16 Burkitt biopsies derived from different donors. Three biopsies reacted weakly. Unless stated otherwise, fluorescein isothiocyanate-conjugated goat anti-human IgG serum (Hyland Laboratories, Los Angeles, Calif.) served for the second stage of the reaction. In all tests, cells were exposed to this reagent alone for control. The results are expressed as the fluorescence index (FI) which is calculated as follows: per cent negative cells in the test sample minus the per cent negative cells in the control divided by the per cent negative cells in the control. Since the controls often revealed very few or no positive cells, the results in these cases can be given also as per cent MIF-positive cells in the test sample.

Anti-EBV Test.—Cells (about $3-4 \times 10^5$ /cover slip) were sedimented at 900 g for 10 min. The supernates were drained off and the cells resuspended in the remaining fluid. A small drop of suspension was placed on a cover slip (6×30 mm), evenly spread, and rapidly dried at 37°C. The preparations were then fixed in acetone at room temperature for 10 min and stored in stoppered test tubes at -20°C until shipped from Stockholm to Philadelphia under dry ice refrigeration.

The procedures for staining by the direct and indirect immunofluorescence techniques have been described (3). Fluorescein isothiocyanate-conjugated human γ -globulin served for direct staining. For the indirect technique human sera with, as well as without, antibodies to EBV were used and goat anti-human IgG conjugates purchased from Hyland Laboratories.

These conjugates which elicited detectable fluorescence when diluted 160-fold, were used in dilutions of 1:20 or 1:30. The percentages of immunofluorescent cells were determined in at least 10 high power fields (750 \times) in various areas of the smear and the average was calculated.

RESULTS

Various established blastoid cell lines of Burkitt tumor or leukemic origins had been tested for membrane immunofluorescence and EBV antigens separately

TABLE I
Comparison of EBV-Positive Cells and Membrane Immunofluorescence in Various Cell Lines

Cell line	Origin	EBV-positive cells (Philadelphia lines)	Membrane immunofluorescence (FI) (Stockholm lines)
		%	
Jijoye*	Burkitt's lymphoma	5-15	0.48
EB3 (14)	" "	3-5	0.29
B35M (15)	" "	2-3	0.52
EB1 (14)	" "	1-3	n.t.
SL1 (16)	" "	1-2	0.37
EB2 (14)	" "	0.5-2	n.t.
Kudi*	" "	0.3-1	0.0
Ogun*	" "	0.1-0.2	0.03
Raji (17)	" "	0	0.07
SKL1 (18)	Leukemia	0.1-0.2	0.0
SKL2 (18)	"	<0.1	0.01
SKL3 (18)	"	0	0.03
SKL4 (18)	"	0	0.0
SKL5 (18)	"	0	0.02
SKL7 (18)	"	0	0.01
LK1D (19)	"	0	0.02
RPMI 7466 (20)	Healthy donor	0	0

* Pulvertaft, J. V. Unpublished observations.

in the Stockholm and Philadelphia laboratories, respectively. While the results obtained with these cell lines (Table I) may not be strictly comparable, since the lines had been maintained in different laboratories under partially different conditions, they nevertheless appeared to indicate that (a) cultures with <0.5% EBV antigen-containing cells as a rule do not yield significant MIF; (b) with rise in EBV-positive cells from 0.5 to up to 15%, the percentages of MIF-positive cells in the corresponding lines increase strikingly; and (c) approximately 10 times more cells show MIF than EBV antigen. Only the Kudi cells do not seem to fit this pattern. It is possible that the percentages of EBV-

positive cells in the Stockholm cultures were lower than in the Philadelphia line. These comparisons suggested that MIF depends not only on the presence of EBV but also upon the extent of the persistent viral infection. In order to confirm this suggestion, strictly comparative tests were carried out.

Recent studies in the Stockholm laboratory had been concentrated upon the establishment of new cell lines from Burkitt tumor biopsies (11) and their MIF reactivities. From cell suspensions used for the MIF tests, a few cover slip smears were made which, after acetone fixation, were sent to Philadelphia for determination of EBV antigen-containing cells. All fixed preparations were stained with human γ -globulin conjugates and the specificity of the fluorescence elicited in given lines was ascertained by the indirect method using sera known to possess antibodies to EBV and others devoid of such antibodies. Lately sera were used for this purpose which were taken from individuals prior to onset of infectious mononucleosis (IM) and again during the acute stage of this disease since recent observations indicate that EBV is one, if not the major cause of IM (21, 22). In every culture which revealed EBV-positive cells by the direct staining methods, indirect tests with EBV-negative or pre-IM sera failed to yield positive results whereas with acute stage sera fluorescence was clearly demonstrable, proving the presence of EBV.

Fig. 1 summarizes results obtained during three different periods in MIF and EBV tests with several recently established Burkitt tumor lines. The data represent the average percentages of positive cells in given lines based upon from 2 to >20 individual MIF tests and from 2-18 EBV tests. The lines were arranged in each observation period according to their descending order of MIF reactivities. It is seen that, with a decrease in MIF-positive cells, the EBV-positive cells also tended to decline but there was no constant ratio between the percentages of positive cells as obtained by the two tests. Indeed, in some instances, high percentages of MIF-positive cells were noted but only relatively low percentages of EBV-positive cells, and conversely, some cultures revealed little MIF but relatively high levels of EBV antigen-containing cells. Furthermore, the order of the cell lines was found to vary from month to month to some extent. This was due in part to addition of new cell lines with high MIF reactivities, and in part also to changes in the reactivities of previously tested cultures. Examples of such variations in given cultures are shown in Fig. 2. The Silfere (A) and Esther (see Fig. 1) lines revealed strong MIF and unchanging EBV reactions throughout the 6-month period of observation. The Annah (B) and Opasa cultures showed a gradual decline of EBV-positive cells which was accompanied to some extent by a decline also of the MIF activity. Newly established cultures of Wilkister (C) or Daudi or Katana revealed significant degrees of MIF from the beginning, that is when sufficient cells became first available for test, whereas EBV-positive cells were rare initially but reached relatively high levels on further maintenance of the

cultures. In another newly established line (Kiliopa, D), MIF and EBV reactions appeared gradually and apparently in parallel. The cultures of Margaret (E) and Ekesa indicate that a relatively high percentage of EBV-positive cells may not necessarily be accompanied by significant degrees of MIF activity. The Margaret line developed significant MIF activity (70%

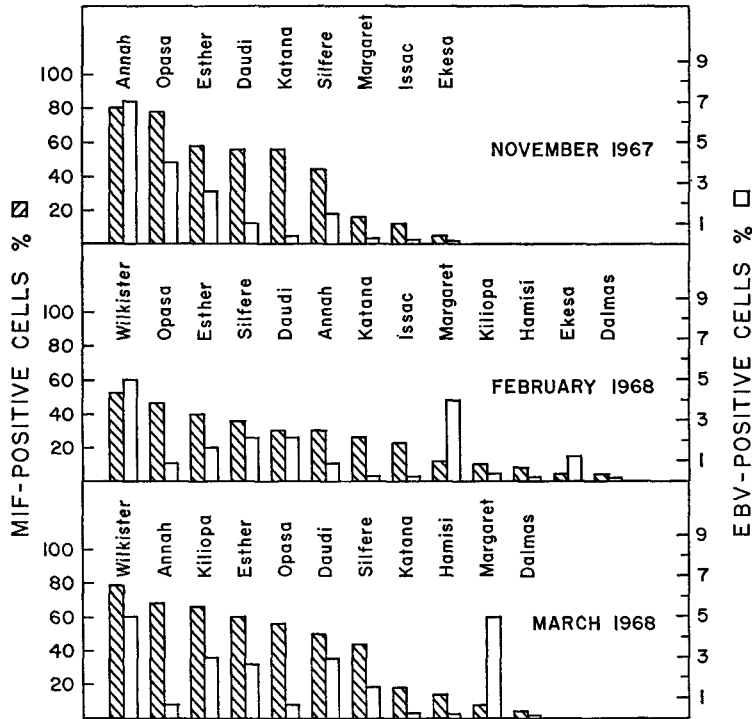


FIG. 1. Comparison of cell membrane and EBV-specific immunofluorescence in recently established cultures of Burkitt tumor cells.

positive cells) after the present experiments were terminated. Finally, cultures of Dalmas (F), Issac, and Hamisi contained only few EBV and MIF-positive cells in the course of these studies.

The observation that early cultures may show strong MIF reactions but may reveal few EBV-positive cells is matched by experience with fresh biopsy cell suspensions. It was shown earlier (2) that many biopsies yielded cells suitable for MIF tests. In contrast, acetone-fixed smears of cells from 35 biopsies revealed EBV-positive cells in only four instances and then no more than 3-5 cells among tens of thousands present per slide. In a few additional biopsies, questionably positive structures were seen which resembled disinte-

grating cells. The scarcity of positive cells and their irregular presence in different smears prevented specific identification of the immunofluorescence as being due to EBV. Table II presents data on biopsy cells on which both MIF and EBV tests were carried out.

The results described indicated that different antigens and antibodies

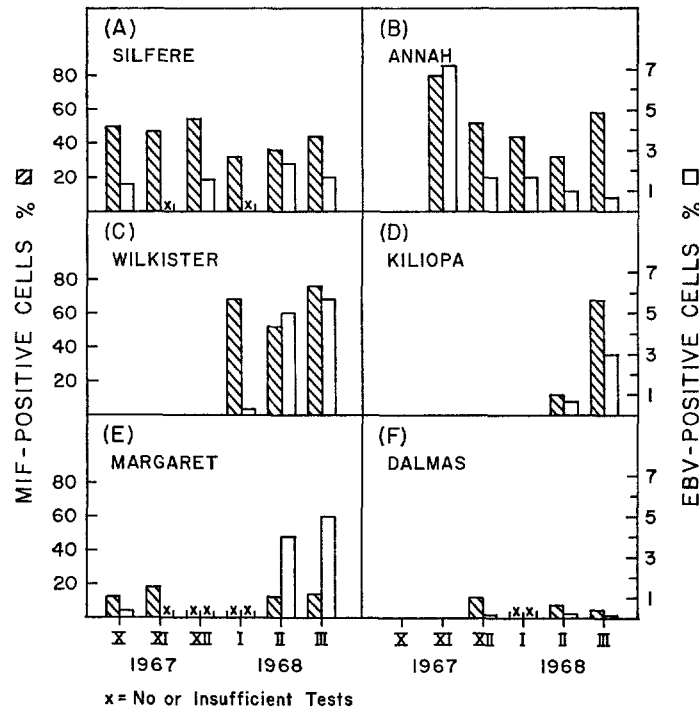


FIG. 2. Patterns of changes in cell membrane and EBV-specific immunofluorescence in the course of maintenance of recently established cultures of Burkitt tumor cells.

participate in the two types of immunofluorescence tests. This became evident also from two preliminary experiments in which the standard Mutua serum was adsorbed with MIF-positive cells of several lines. As a consequence of this procedure, the serum induced now only insignificant MIF reactions in appropriate test cultures whereas the titer of antibodies to EBV was not affected.

DISCUSSION

The various observations suggest that in cultures of Burkitt tumor cells, whether of long standing or recently initiated, the detection of membrane fluorescence may depend not only upon the presence of EB virus but to a considerable degree also upon the extent of the persistent EBV infection. Generally,

many more cells showed membrane fluorescence than EBV antigen but there was no constant ratio between the percentages of cells which were reactive in the two tests. Furthermore, the two reactivities in given cultures did not remain necessarily at constant levels as time progressed but showed increases or decreases, often apparently independent of each other. Nevertheless, when more than 0.5% of the cells in well-established cultures revealed the presence of EBV antigen, the percentages of cells with membrane fluorescence were

TABLE II
Comparison of MIF and EBV Reactions of Cells from Fresh Burkitt Tumor Biopsies

Patient	Age	Sex	KCC No.	Date of biopsy	MIF reaction		EBV reaction					
					Reagent*	FI	Result	No. of + or (?) cells/smear				
Kimone	8	M	813	11/14/67	a	0.48	—	0				
	Ekesa	M	816	11/14/67	b	0	—	0				
2/21/68				a	0.14	?	(1)					
				d	0.10							
Malakwe				8	M	820	11/21/68	a	0.71	—	0	
Wilkister	7	F	819	12/6/67	a	0.39	+	1-2				
				b	0.67							
Juma	8	M	827	1/13/68	a	0.54	?	(2)				
				1/26/68	b	0.38						
Opasa	5	M	766	1/13/68	a	0.33, 0.48	?	(5)				
					2/21/68	a	0.45	+	3-4			
				Fanis	10	F	300	1/19/68	a	0.39	+	2-3
								c	0.42			

* a = goat anti-human kappa chain conjugate (Hyland Laboratories), b = rabbit anti-human IgG (G. Goldstein), c = goat anti-human γ globulin (Baltimore Biological Laboratories, Baltimore, Md.), d = goat anti-human IgG (Hyland Laboratories).

usually high and, conversely, when the EBV-positive cells were of very low frequency, membrane immunofluorescence was, as a rule, negligible. There were, however, a number of exceptions. Young cultures, and especially suspensions of fresh biopsy cells may yield high degrees of MIF in the presence of few or no EBV-positive cells. Conversely, older cultures may occasionally show relatively large numbers of EBV antigen-producing cells in the absence of significant MIF activity. These observations imply that at least two distinct antigens are involved in the two types of tests. This is further supported by the fact, to be reported in a subsequent paper of this series, that sera can be found which are highly reactive in the MIF test but show only low titers against EBV and vice versa.

The fact that fresh biopsy cells or young cultures may show significant MIF activity but few or no EBV-positive cells might have two reasons: (a) Burkitt tumor patients generally have high levels of antibodies to EBV (3, 7, 21) and some antibodies are likely to be carried over into the cultures. These antibodies may restrict the spread of the infection until diluted out in the course of maintenance of the cultures and, since the virus-producing cells usually show varying degrees of degeneration, it is conceivable, in addition, that antibody enters and blocks the EBV-staining reactions. Antibodies to cell membrane antigens are also present in most patients' sera but the antigenic mass in tumors may exceed the capacity of available antibodies to block all surface antigen. However, γ -globulins have been detected on biopsy cells (23) and their presence could conceivably account for negative MIF tests with some of the biopsy cell suspension. (b) MIF-positive cells generally exceed EBV-positive cells by a substantial factor. If the virus were responsible for inducing the membrane antigens, many more cells must be invaded by the virus than actually synthesize viral capsid antigens. Some support for "nonproductive" cellular infections may be derived from the effect of arginine-deficient media upon the persistent EBV infection in Burkitt tumor cell cultures (6). Such media prevent cellular divisions but enhance the percentage of EBV antigen-producing cells by a factor of about 10. This observation has been interpreted to denote that the arginine deficiency removed an intracellular block of viral replication while enough arginine remains available within the cells for production of EB viral coat proteins. It is almost certain that arginine is required for this purpose as it is for synthesis of capsid proteins of other members of the herpes group (24-26). The assumption, that only about 1 in 10 invaded cells supports EBV replication, could account for the difference in the percentages of EBV- and MIF-positive cells if one postulates that the viral genome in the other 9 cells is not entirely dormant but induces cellular surface changes which are reflected in the membrane immunofluorescence reaction.

The reverse finding, that some cultures reveal relatively high levels of EBV-positive cells but no significant MIF activity is less readily explained. It has been noted, however, that the presence and concentration of membrane antigens depends to some extent upon the age of the cultures after the last feeding and whether or not certain antibiotics (kanamycin) are present in the culture media (unpublished observations). Proteins are apparently shed from the cell surface under certain conditions which could account for loss of MIF activity. This possibility remains to be explored.

SUMMARY

A comparison was made of the immunofluorescence tests for detection of cell membrane and Epstein-Barr virus antigens in cells from Burkitt tumor biopsies or continuous cultures derived therefrom. On the whole, cell membrane fluores-

cence in established lines appeared to depend not only upon the presence of EBV but to a considerable degree also upon the extent of the persistent viral infection. There was no constant relationship, however, between the results of the two tests and exceptions to the rule were noted. These observations indicate that different antigens are involved in the two tests. Biopsy cells in general and young cultures may reveal strong MIF activity but few, if any, EBV-positive cells. The reverse, the presence of relatively large numbers of EBV antigen-containing cells in the absence of significant MIF reactions, was also noted on occasion in a few established cultures. The possible interpretations of these findings have been discussed.

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