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REACTIVITY OF RADIOIODINATED SERUM ANTIBODY FROM BURKITT'S LYMPHOMA AND NASOPHARYNGEAL CARCINOMA PATIENTS AGAINST CULTURE LINES DERIVED FROM BURKITT'S LYMPHOMA

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SUMMARY

The IgG serum immunoglobulin fraction of two Burkitt's lymphoma (Mutua and Kiliopa) and one African nasopharyngeal carcinoma patient (Kipkoech) was conjugated to iodine-131 (^{131}I). It is known from previous studies with fluorescein labelled conjugates that all three sera contain antibody against the Epstein–Barr virus (EBV)-associated membrane antigen complex, present on the surface of lymphoblastoid cells in EBV-carrier cultures. All three radioiodinated conjugates attached to live cells of an EBV-carrying Burkitt line (Maku), but not to EBV-free Raji cells. A Swedish control serum (Berith) did not block the binding of any of the three conjugates, whereas unconjugated sera of Mutua, Kiliopa and Kipkoech showed various degrees of blocking and cross-blocking. The blocking patterns were in good agreement with previous tests, performed with the same sera against their fluorescein conjugated derivatives.

Antibody release tests, involving preincubation of live cells with one of the three conjugates, followed by incubation with unlabelled serum revealed a certain 'hierarchy' between the three sera with regard to their ability to displace radioiodinated surface-coupled immunoglobulin. This ability could be related to the competitive behaviour of the same sera in the cross blocking tests. The results are believed to reflect differences in the affinity of the three antibodies, due either to differences in fit in relation to the surface antigen(s) carried by the Maku target cell, or to differences in the duration of immunization in the three patients.

INTRODUCTION

Distinctive, cell membrane associated antigens have been demonstrated on Burkitt lymphoma biopsy cells (Klein *et al.*, 1966, 1967) and derived tissue culture lines (Klein *et al.*, 1967, 1968, 1969) by direct and indirect membrane fluorescence. The membrane antigen complex was associated with the presence of the herpes-like Epstein–Barr virus (EBV).

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It could not be demonstrated on EBV-free cell lines and lines with only a low proportion (less than 1%) of EBV-positive cells (Klein *et al.*, 1969). The presence or absence, as well as the level of antibodies directed against the intracellular EBV-antigen complex and the membrane associated system, respectively, was concordant in the majority (about 80%) of the sera tested. The existence of a 'discordant' minority, as well as absorption experiments nevertheless showed that the two antigen complexes are distinct (Pearson *et al.*, 1969).

As a rule, membrane reactive antibodies were localized in the IgG fraction of positive sera (Smith *et al.*, 1967). In addition to membrane fluorescence, they could be demonstrated by the method of C1 transfer and immune adherence as well (Nishioka *et al.*, 1968).

By fluorescein conjugation of IgG from reactive sera, the indirect membrane fluorescence test was converted into a direct test (Goldstein *et al.*, 1969; Klein *et al.*, 1969). A number of reference conjugates, derived from donors with various clinical conditions, including Burkitt's lymphoma and nasopharyngeal carcinoma, or from healthy individuals, were tested for blocking and cross-blocking against the corresponding unconjugated sera (Svedmyr *et al.*, 1969). The majority of the combinations showed a symmetrical behaviour in reciprocal tests, but there were some notable exceptions. In certain serum–conjugate pairs, blocking was only obtained in one direction and the reciprocal combination failed to block or showed only partial blocking (Svedmyr *et al.*, 1969). It was tentatively assumed that different sera contained different numbers of antibody components, directed against different receptors within the membrane antigen complex. Alternatively, asymmetrical blocking may be due to a difference in antibody affinity towards the same antigenic receptors. The present investigation is an attempt to study this question further, by using radioiodine-labelled antibodies and combining blocking with antibody release tests.

MATERIALS AND METHODS

Tissue culture lines

Two established lymphoblastoid cell lines were used, both derived from Burkitt's lymphoma. The cell line Maku was established from a biopsy preparation received from Nairobi (Nadkarni *et al.*, 1969); it is strongly reactive in the EBV-associated membrane immunofluorescence test (Yata & Klein, 1969). The other cell line, Raji, has been derived from a Nigerian case of Burkitt's lymphoma in 1963 (Pulvertaft, 1964); it is negative in membrane immunofluorescence (Klein *et al.*, 1967) and it carries no EB-virus, according to electron microscopy and intracellular immunofluorescence.

In preparation for the present tests, 8×10^6 cells were added to 20 ml of Eagle's Basal Medium with 20% foetal calf serum. The cultures were kept stationary at 37°C in a 5% CO_2 atmosphere, in loosely screw-capped bottles and were fed every 3rd day by replacing two-thirds of the volume with fresh medium. At each feeding, the number of cells was adjusted to 8×10^6 per bottle. The resulting cell harvest was supplied every third day for the tests; it contained 80–90% living cells, as a rule. After washing them twice with K-glucose GVB^{++}, the cells were adjusted to a concentration of 1×10^6/ml.

Sera

Four sera were used; they have all been characterized previously with regard to their anti-EBV and membrane reactivity (Smith *et al.*, 1967; Klein *et al.*, 1967, 1969; Pearson *et*

al., 1969; Svedmyr *et al.*, 1969), including blocking and cross-blocking tests. Two, Mutua (Kenya Cancer Council No. KCC 454) and Kiliopa (KCC 834) were derived from Burkitt lymphoma cases, the former in long term regression, the latter in the course of progressive tumour growth. One serum was derived from a patient with nasopharyngeal carcinoma, Kipkoech (KCC 883). These three sera were received from Mr Peter Clifford, Kenyatta National Hospital, Nairobi, in dry ice. A fourth serum, 'Berith', was taken from a healthy Swedish technician, and used as an anti-EBV and membrane fluorescence negative control. All sera were inactivated at 56°C for 45 min, distributed in 1-ml amounts into small tubes and stored at −20°C until use.

DEAE-cellulose column chromatography

Two-millilitre serum aliquots were dialysed for 12 hr at 4°C against several changes of 1 litre of 0·005 M phosphate buffer, pH 8·0. The material was passed through a 1·5 × 10 cm DEAE-cellulose column equilibrated with the same buffer and washed out with the same buffer. The flow rate was 30 ml/hr. Four millilitre aliquots of the eluate was collected in a tube, and the protein was checked by a Leitz spectrophotometer. The pooled eluate was concentrated by ultrafiltration and dialysed against 0·145 M saline or 0·15 M phosphate buffer, pH 7·6. Immunodiffusion and immunoelectrophoresis of the final product revealed a single line with goat anti-human serum and with goat antiserum to human IgG.

Labelling

Labelling was carried out by the chloramin T iodination technique. Four milligrams of IgG dissolved in 1 ml 0·15 M phosphate buffer, pH 7·6, was used for iodination. Iodine-131, carrier free, without reducing agent added, was provided by the Radiochemical Centre, Amersham, England. As a rule, the IgG preparations had a mean iodine content of 1 atom per IgG molecule. The samples were counted in an automatic scintillation detector with a well in thallium activated NaI crystal (Tracerlab type 51). The results were printed out on paper tape. Labelled IgG was diluted 1:9 with non-labelled IgG of the same type before use.

Blocking tests

Two million cells were added in 0·02-ml volumes to small tubes containing 0·2 ml 1:2 diluted serum. The mixture was incubated in a 37°C water bath for 30 min with intermittent shaking. After incubation, the cells were washed three times with K-glucose GVB^{++} and incubated at 37°C for another 30 min with 0·2 ml ^{131}I-labelled IgG ([^{131}I]IgG) in serial two-fold dilutions. After washing three times with the same buffer, the radioactivity of the sediments were counted.

Antibody release test

Aliquots (0·02 ml) from cell suspensions containing 1×10^7 cells/ml were mixed with 0·2 ml [^{131}I]IgG solution. The mixture was incubated at 37°C for 30 min and washed three times with K-glucose GVB^{++}. After the addition of 0·2 ml serum, in serial two-fold dilutions, the cells were incubated again at 37°C for 30 min. After centrifugation, 0·1 ml of the supernatant was precisely calibrated and its radioactivity was measured.

RESULTS

Binding of radioiodinated immunoglobulins to the target cells

Fig. 1 shows the relationship between the amounts of radioactive Mutua, Kiliopa, and Kipkoech IgG, respectively, added to the reaction mixture and the number of counts attached to the Maku target cells. In this experiment, 2×10^6 cells, suspended in 0·02-ml

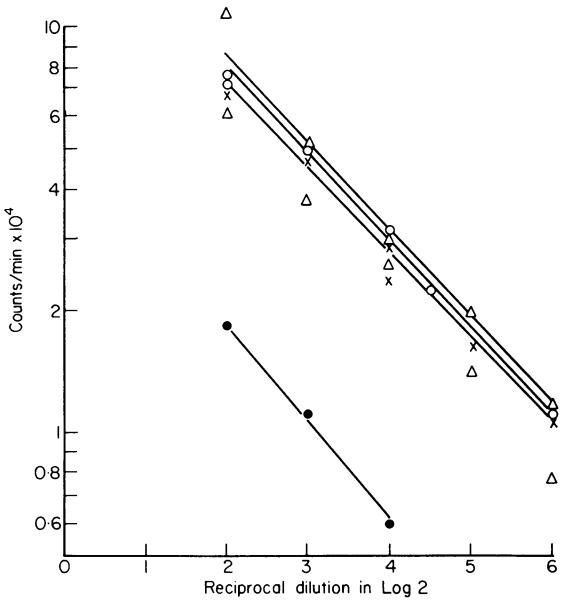

FIG. 1. Reactivity of three radioiodinated IgG preparations against the Maku and the Raji target cells. Radioiodinated IgG tested against the Maku target cell: △, Kiliopa IgG; X, Kipkoech IgG; ○, Mutua IgG. Radioiodinated IgG tested against Raji target cells: ●, Kipkoech IgG. $1/n = 9{\cdot}4$.

TABLE 1. Reactivity of three IgG preparations against the Maku target cell

	Bound IgG (CPM)			% bound IgG		
Dilution	Mutua	Kiliopa	Kipkoech	Mutua	Kiliopa	Kipkoech
1	72750	62418	67165	2·9	2·4	2·6
2	50259	52240	46537	3·9	4·1	3·6
4	31700	29972	32095	5·0	4·7	5·0
8	22756	20656	16214	7·1	6·5	5·1
16	10879	12883	11905	6·8	8·1	7·5

volumes, were mixed with 0·2 ml of the serial two-fold IgG dilutions. After incubation at 37°C for 30 min, followed by centrifugation, the radioactivity fixed to the cells was counted. All three immunoglobulin preparations gave a linear relationship with a slope of −9·4. With the same amount of IgG, the antibody activity of the three sera was approximately

equal. Table 1 shows the percent IgG bound with different dilutions of the three immunoglobulin preparations. The radioactivity bound to the Raji target cell was also measured, after exposure to the Mutua, Kiliopa and Kipkoech conjugates, respectively. It was only about 10–20% of the radioactivity bound to the Maku target cell, similar for all three conjugates.

TABLE 2. Reactivity of radioiodinated Muta IgG against live and dead Maku cells

Dilution of Mutua IgG	Bound Mutua IgG CPM		% bound Muta IgG	
	Live culture	Dead culture	Live culture	Dead culture
2	50,259	58,519	3·9	4·5
4	31,700	38,930	5·0	6·1
8	22,756	21,584	7·1	6·7

TABLE 3. Blocking effect of unconjugated serum from Berith (control), Mutua (BL*), Kiliopa (BL*), and Kipkoech (NPC†) against radioiodinated IgG of Mutua, Kiliopa and Kipkoech, respectively‡

Unconjugated blocking serum	Radioiodinated IgG of:											
	Mutua, reagent dilution				Kiliopa, reagent dilution				Kipkoech, reagent dilution			
	2	4	8	16	2	4	8	16	2	4	8	16
Berith	5	0	0	0	0	0	0	0	0	0	0	0
Mutua	56	62	65	89	38	54	66	76	28	37	38	39
Kiliopa	52	64	73	81	48	61	76	84	26	28	41	67
Kipkoech	49	58	74	87	55	63	70	76	50	63	72	78

* Burkitt's lymphoma.

† Nasopharyngeal carcinoma.

‡ The figures designate the radioactivity bound by the cells pretreated with the blocking serum, subtracted from the radioactivity of the cells exposed to the conjugate without blocking serum, divided by the latter figure and multiplied by 100.

The question arose whether most of the radioactivity bound to the Maku target cell reflects binding to surface antigen receptors, present on the outer membrane of viable cells. Dead cells may introduce important sources of error, due to defects in the cell membrane. In the fluorescence test against viable target cells, the conjugates diffuse freely across dead cells, resulting in a homogeneous, non-specific fluorescence. In order to assess whether the presence of dead cells would influence the binding of radioiodine labelled antibody to an important extent, the binding of radioiodine-labelled Mutua IgG by living and dead Maku cells was compared. The dead cells were stored in the refrigerator in GVB, until nearly the entire cell population became stainable by trypan blue. The results are shown in Table 2.

It will be seen that slightly higher counts were obtained with the dead cell suspension, but the difference was relatively minor. It is possible, however, that the radioactivity taken up by non-specific penetration into the dead cells was counteracted by the degradation of surface antigens.

Since dead cells constituted a relatively small fraction of the cell population in all tests performed in this study, no attempt was made to introduce any correction and the data were calculated as if the cell suspension only contained viable cells.

Blocking tests

The results of the blocking tests, performed with radioiodinated Mutua, Kiliopa or Kipkoech IgG, and unconjugated control serum (Berith) or Burkitt lymphoma serum (Mutua or Kiliopa) or nasopharyngeal carcinoma serum (Kipkoech) are shown in Table 3. Percentage blocking was calculated as indicated in the footnote of the table. Berith's serum

TABLE 4. Comparison of blocking against flourescein conjugated and radioiodinated Muta, Kiliopa and Kipkoech IgG, respectively, by whole undiluted serum from the same donors

Blocking serum	Conjugated immunoglobulin:	Mutua	Kiliopa	Kipkoech
Mutua	FBI	93	70	57
	RBP	89	76	39
	FBI/RBP ratio	1·04	0·92	1·46
Kiliopa	FBI	86	80	50
	RBP	81	84	67
	FBI/RBP ratio	1·06	0·95	0·75
Kipkoech	FBI	82	80	83
	RBP	87	76	78
	FBI/RBP ratio	0·94	1·05	1·06

FBI, Blocking of direct membrane fluorescence expressed by the blocking index (Svedmyr *et al.*, 1969) × 100; RBP, percentage blocking against the radioiodinated conjugate, dilution 1:16.

did not block any of the three immunoglobulins at any dilution tested. Mutua's serum blocked the binding of Mutua's IgG between 56 and 89%, depending on the reagent dilution. Against Kiliopa's labelled IgG, the blocking effect of Mutua's serum ranged from 38 to 76%. Against the radioiodinated Kipkoech's IgG, the blocking effect ranged from 28 to 39%. The blocking effect of Kiliopa's serum against radioiodinated Mutua IgG was nearly identical to the effect of Mutua's serum against its own conjugate when tested for blocking against Kiliopa's IgG, Kiliopa's serum gave a somewhat better effect (ranging from 48 to 84%) than Mutua's serum (38 to 76%). Both Kiliopa's and Mutua's serum gave approximately equal blocking effects against radioiodinated Kipkoech IgG, except the highest reagent dilution (1:16) that was blocked more efficiently by Kiliopa than by Mutua serum.

The Kipkoech serum gave a high blocking effect against the Mutua conjugate (49 to 87%), the Kiliopa conjugate (55 to 76%) and the Kipkoech conjugate as well (50 to 78%).

The cross-blocking tests thus show that the control (Berith) serum could not block any of the three radioiodinated immunoglobulins. Mutua's serum showed the best blocking effect with his own immunoglobulin and its efficiency decreased towards Kiliopa and Kipkoech IgG, in this order. Kiliopa's serum was intermediate, in showing good blocking against both Mutua and Kiliopa IgG, but it fell short of blocking Kipkoech IgG. The Kipkoech serum was superior to the others since it blocked all three immunoglobulin conjugates with a high efficiency.

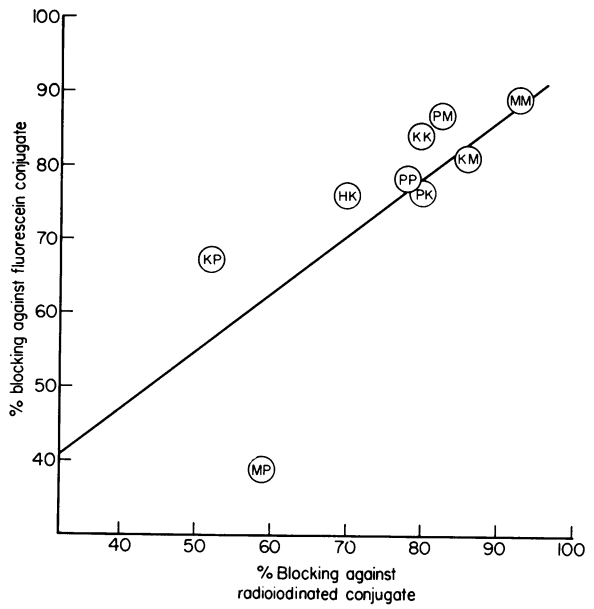

FIG. 2. Relationship between the blocking test against fluorescein conjugated and radioiodinated IgG, respectively. In each circle, the first letter denotes the source of the unconjugated serum and the second the immunoglobulin conjugate: M, Mutua; K, Kiliopa; P, Kipkoech. $r = 0{\cdot}78$; $P \cong 0{\cdot}01$.

The same three sera and their fluorescein-conjugated derivatives have been tested previously by blocking and cross blocking of direct membrane fluorescence (Svedmyr *et al.*, 1969). It was of interest to compare the present results with the corresponding values obtained with the same sera in the fluorescence test. The 1:16 dilution of the radioiodine conjugate which contained the same amount of IgG as used in fluorescence blocking test, was chosen for this comparison. The results are shown in Table 4 and Fig. 2. There was an excellent correlation between the two tests.

Antibody release tests

The results are shown in Table 5. The figures are based on the radioactivity found in the supernatant after incubating the cells, preexposed to radioiodinated IgG, without adding any serum. The counts obtained with the three IgG preparations were nearly identical. The efficiency of the three sera in releasing the radioiodinated IgG attached to the cells was compared by calculating a 'release index', defined as the ratio between the radioactivity

TABLE 5. Summary of antibody release tests

Serum		Radioiodinated IgG		
Designation	Dilution	Mutua	Kiliopa	Kipkoech
GVB^{++}	—	1·00	1·00	1·00
Berith	2	1·61	1·61	1·61
Mutua	2	2·86	2·48	2·50
	4	2·59	2·34	2·33
	8	2·48	2·25	2·23
	16	2·25	2·15	2·16
	32	2·19	2·05	2·06
Kiliopa	2	3·70	3·14	2·80
	4	3·35	2·97	2·54
	8	3·30	2·80	2·53
	16	3·12	2·68	2·41
	32	2·83	2·52	2·25
Kipkoech	2	3·85	3·70	2·84
	4	3·60	3·53	2·55
	8	3·47	3·33	2·16
	16	3·12	3·10	1·97
	32	3·00	2·97	1·62

The 'release index' was calculated as follows: the radioactivity of the supernatant after incubation with the test serum was divided by the radioactivity of the supernatant following incubation with buffer only.

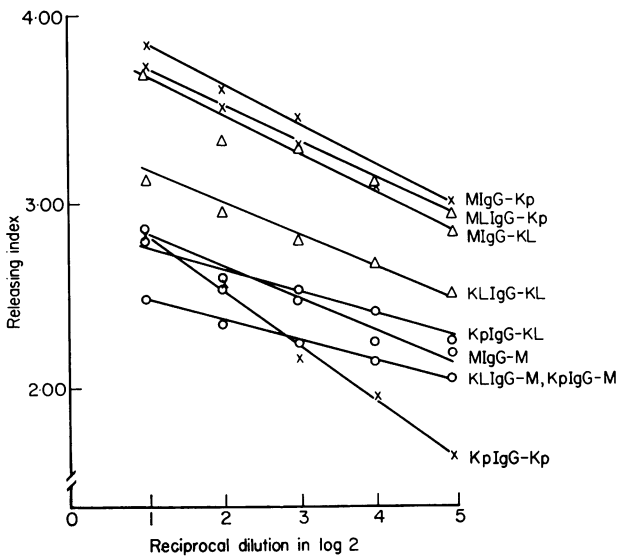

FIG. 3. Antibody releasing test. The symbols on the right of each curve designate the radioiodinated IgG reacted with the cells, followed by the unconjugated serum. Kl, Kiliopa; Kp, Kipkoech; M, Mutua.

found in the supernatant of the serum-exposed sample, and the control sample, incubated with buffer alone.

The addition of the control serum (Berith) at the dilution of 1:2 gave a positive release index of 1·61. Since this serum is not known to contain any antibodies against the EBV-associated membrane–antigen complex and does not block any of the three conjugates (Klein *et al.*, 1969; Pearson *et al.*, 1969), it is assumed that some non-specific serum action is responsible for this effect. Mutua's serum released considerably more radioactivity from cells coated with all three conjugates. Its release index towards Mutua's IgG ranged from 2·86 to 2·19 with increasing serum dilutions (1:2–32, cp. Table 5). Slightly lower release indexes were obtained against Kiliopa and Kipkoech IgG, with nearly identical values for these two conjugates. Kiliopa's serum was more powerful against the Mutua conjugate, with a releasing index that ranged from 3·70 to 2·83. It was also stronger against Kiliopa's IgG than Mutua's serum, although it released less radioactivity (range: 3·14–2·52) than from the cells coated with Mutua IgG. Against cells coated with Kipkoech's IgG, the Kiliopa serum dilutions were only slightly more efficient than the corresponding Mutua serum dilutions (ranging from 2·80 to 2·25). Kipkoech's serum was the most powerful releaser of Mutua's IgG (3·85 to 3·00) and of Kiliopa's IgG as well (3·70 to 2·97) whereas its effect on cells coated with Kipkoech's own IgG was not consistently superior to the effect of the two other sera (2·84 to 1·62).

If the figures in Table 5 are considered in relation to the releasing effect of each serum on its own corresponding IgG conjugate, it will be seen that Mutua releases its own conjugate best, Kiliopa releases Mutua's conjugate better and Kipkoech's conjugate less well than its own, whereas Kipkoech's serum releases Mutua IgG best, Kiliopa IgG nearly as well, but its own IgG much less well. There is thus a certain 'displacement hierarchy' between the three sera, going from Mutua, through Kiliopa, to Kipkoech, also illustrated in Fig. 3. A similar hierarchy was found in the blocking test, as described above in detail.

DISCUSSION

In blocking tests against fluorescein conjugated immunoglobulin derived from the same sera as used in the present investigation, it was found (Svedmyr *et al.*, 1969) that unconjugated Mutua serum completely blocked the membrane reactivity of EBV-carrying lymphoblastoid cell lines with Mutua's IgG conjugate, but showed only partial blocking of the Kipkoech's conjugate. In contrast, the unconjugated Kipkoech serum blocked the Mutua and the Kipkoech conjugates as well. Kiliopa's serum showed an intermediate position: it blocked the Mutua and the Kiliopa conjugate, but showed only partial blocking against the Kipkoech conjugate; this was similar to the behaviour of the Mutua serum, but Kiliopa showed regularly a better blocking activity against the Mutua conjugate than the reciprocal test, Mutua's serum against Kiliopa conjugate.

Two alternative explanations can be considered for the asymmetric blocking patterns observed. Conceivably, the EBV-associated membrane–antigen complex may consist of more than one site and some sera—Mutua—would contain antibodies against a smaller number of components than others, like Kipkoech. Kiliopa would be intermediate. Alternatively, only one site would exist, but the antibodies found in different sera would differ in their affinity for the site. This would imply that Kiliopa's antibody would have higher affinity than Mutua's and Kipkoech's antibody would be even higher. It may be relevant in

this connection that one of us (Inoue, 1966) has previously shown that cross-reacting antigens can be dissociated from the antibody portion of antigen–antibody complexes by the addition of homologous antigen.

When homologous antigen is added to a complex of antibody directed against itself and a cross-reacting antigen, the cross-reactive antigen dissociates from the antibody portion of the complex and the genuine antigen combined with the antibody.

Blocking tests reported in the present paper, using radioiodinated IgG fully confirmed the previous findings with fluorescein conjugated antibody. The percentage blocking of radioiodinated antibody binding showed a good quantitative correlation with the percentage of cells blocked according to direct membrane fluorescence. The 'blocking hierarchy' of the three sera was the same in both tests, with Mutua at the bottom and Kipkoech at the top. Kiliopa continued to occupy an intermediate position.

Extensive cross-blocking tests with these and a number of other conjugates (Svedmyr *et al.*, 1969) have been previously interpreted to mean that the EBV-associated membrane-antigen had a complex structure, with a number of different antigenic sites, and that some sera contained antibodies against a larger number of components than others. Mutua's serum was believed to contain at least two antibodies, because a number of sera derived from healthy Burkitt patients' relatives, many of them negative in the ordinary anti-EBV test, did react with the membrane antigen(s), did not react with antigen-negative cells, and showed cross-blocking against each other, but not against Mutua's serum. Mutua's serum blocked all of them (Pearson *et al.*, 1969; Svedmyr *et al.*, 1969).

In the present study, the release of cell-attached radioiodinated IgG upon exposure to unconjugated sera was used to study the ability of the various immunoglobulins to displace each other. Substantial antibody release could be demonstrated by this procedure and the 'releasing power' of the different sera paralleled their blocking potency. Mutua's serum released Mutua's IgG best; Kiliopa's serum released more Mutua IgG and less Kipkoech IgG than its own IgG; and Kipkoech's serum released more IgG of the other two donors than of its own. The most probable interpretation is that the three sera contain antibodies with different degrees of affinity towards the surface antigen(s) present on Maku cells, and that lower affinity antibodies can be displaced from the Maku target cell surface by higher affinity antibody.

Two alternative interpretations can be advanced to account for the differences in the antibody affinity in the three patients. Conceivably, the membrane antigens present on the original cells that have elicited antibody formation in the three patients could be slightly different. More specifically, this would mean that the membrane antigen expressed on the autochthonous immunogenic cells, responsible for the primary immunization of Mutua, Kiliopa and Kipkoech would have an increasingly closer fit with the antigen(s) present on the Maku target cell, in this order. Alternatively, it is possible that all three antibodies are directed against the same site, and the increasing affinity could be due to a more prolonged immunization in the 'higher affinity' sera. It is noteworthy in this connection that Mutua's serum has been derived from a Burkitt lymphoma patient in long term regression, several years after the tumour has disappeared, leaving no clinical signs of residual disease (Klein *et al.*, 1967). Kiliopa, on the other hand, was a Burkitt patient with progressively growing tumour, at the time of the serum sampling, and the same was true for the nasopharyngeal carcinoma of Kipkoech. The disease history of Kipkoech was longer than of Kiliopa.

Since only one target cell (Maku) was used in the present investigation, in contrast to

some of the previous studies (Klein *et al.*, 1969; Svedmyr *et al.*, 1969), where several target cells have been used, a clarification of this question will have to await further investigation. It is also too early to say whether the relationship between the anti-EBV negative, membrane-positive Burkitt relative sera (Klein *et al.*, 1969), and particularly their asymmetrical blocking relationship to Mutua's serum, mentioned above, can be explained as another difference in affinity, or will continue to show the existence of two distinct antigenic sites. In preliminary antibody release tests using fluorescein conjugated IgG (Svedmyr *et al.*, 1969) no evidence was obtained for the hypothesis of affinity difference, but the fluorescence technique may not be sufficiently sensitive and quantitative to reveal this.

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