Clin. exp. Immunol. (1969) **5**, 443–459.

EPSTEIN–BARR VIRUS-ASSOCIATED ANTIBODY PATTERNS IN CARCINOMA OF THE POST-NASAL SPACE

A. DE SCHRYVER, S. FRIBERG, JR, G. KLEIN, W. HENLE; G. HENLE, G. DE-THÉ, P. CLIFFORD, AND H. C. HO

Department of Tumor Biology, Karolinska Institutet Medical School, Stockholm; The Children's Hospital of Philadelphia and School of Medicine, University of Pennsylvania, Philadelphia; Centre International de Recherche sur le Cancer, Lyon; Kenyatta National Hospital, Nairobi and the Royal Hong Kong Jockey Club, Institute of Radiology, Queen Elizabeth Hospital, Kowloon.

(*Received* 7 *May* 1969)

SUMMARY

Sera from African patients with tumours of the post-nasal space, Hong Kong Chinese patients with carcinomas of the post-nasal space and from Indian donors with buccal, oro- and hypopharyngeal carcinomas, as well as African control sera from healthy persons and individuals with various non-neoplastic and neoplastic diseases were tested for anti-Epstein–Barr virus titres and for antibodies capable of blocking the direct membrane immunofluorescence reaction obtained between Epstein–Barr virus carrying lymphoblastoid cell lines and two different fluorescein conjugated reference sera; one from a patient with Burkitt lymphoma (F-Mutua conjugate) and the other from a patient with carcinoma of the post-nasal space (F-Kipkoech conjugate). Ninety per cent or more of the African and Chinese patients with carcinoma of the post-nasal space gave at least one high test, and 60% were high in all three assays. In contrast, African patients with post-nasal space tumours other than carcinomas, African controls and Indian buccal, oro- and hypopharyngeal carcinomas gave high reactions in all three assays in less than 20%.

INTRODUCTION

High levels of antibodies to the Epstein–Barr virus (EBV), a member of the herpes group, were found to be associated thus far with three diseases: Burkitt lymphoma (BL), infectious mononucleosis (IM) and carcinoma of the post-nasal space (CaPNS). The sera from by now well over 100 patients with histologically confirmed Burkitt lymphoma have all been found to contain antibodies to EBV, as a rule to high titres (geometric mean 1:320)

Correspondence: Professor G. Klein, Department of Tumor Biology, Karolinska Institutet Medical School, S-10401 Stockholm 60, Sweden.

(Henle & Henle, 1966; Levy & Henle, 1966). Lower titres were observed in some patients shortly before death or in some long term survivors (Henle *et al.*, in preparation). In a prospective study, classical infectious mononucleosis was shown to occur only among individuals who had no antibodies to EBV previously. During the incubation period and early acute stage of the disease antibodies regularly developed but the peak titres attained were generally somewhat lower than those seen in Burkitt lymphoma and they appear to persist at detectable levels for years and even decades (Henle, Henle & Diehl, 1968; Niederman *et al.*, 1968). Concurrent with the emergence of anti-EBV, other antibodies appeared, capable of interacting with the surface of Burkitt tumour cells and other lymphoblastoid cells from EBV carrier cultures in indirect or blocking of direct immunofluorescence tests (Klein *et al.*, 1968). These antibodies appeared to be the same as the membrane reactive antibodies found in sera of patients with Burkitt tumour (Klein *et al.*, 1967). The majority of the sera of the tested patients with CaPNS possessed antibodies to EBV in titres similar to those seen in Burkitt tumour patients (Henle *et al.*, 1968, and to be published) and they were capable of blocking the direct membrane immunofluorescence reaction as well (Klein *et al.*, 1969). A further parallel was found between BL and CaPNS with regard to the precipitin test with soluble antigen extracts from EBV-carrier cultures described by Old *et al.* (1966, 1968). Sera from patients with these two malignancies were reactive in this test in a high proportion, in contrast to sera from patients with other neoplastic and non-neoplastic diseases.

Carcinomas of the post-nasal space occur at a high frequency in certain geographical regions of the world and in certain ethnic groups (Muir, 1962; Clifford, 1965; Ho, 1967a). The purpose of the present study was to compare the EBV-related antibody patterns with the primary sites and the histopathological appearance of the tumours in representative groups of patients; i.e. East African and Hong Kong Chinese patients with nasopharyngeal tumours (mainly carcinomas), and Calcutta Indian patients with buccal, oro- and hypopharyngeal, rather than nasopharyngeal carcinomas. Control sera from African donors in good health or with non-neoplastic or with other neoplastic diseases were used for comparison. Three serological tests were performed: (a) the anti-EBV immunofluorescence tests using fixed cell smears from EBV-carrying lymphoblastoid cell lines (Henle & Henle, 1966); (b) blocking by test sera of the direct membrane fluorescence induced in EBV-carrying lymphoblastoid cell lines by a fluorescein-conjugated Burkitt tumour patient reference serum (F-Mutua); and (c) the same type of test using a fluorescein conjugated reference serum of an African CaPNS patient (F-Kipkoech).

MATERIALS AND METHODS

Cells

The establishment and maintenance of the cell lines from Burkitt tumour biopsies and peripheral leucocytes of infectious mononucleosis patients have been described in detail (Diehl *et al.*, 1968; Nadkarni *et al.*, 1969). The following EBV carrying cell lines were used:

Two clonal sublines (HR-5 and HR-1-K) of the Jijoye line of Burkitt tumour origin (Hinuma *et al.*, 1967), the Kaplan line, derived from a case of infectious mononucleosis; and a number of cell lines established from Burkitt lymphoma biopsies, received from Kenya, namely NK-9 (Silfere), NK-8 (Esther), NK-6b (Annah), NK-14 (Opasa) and NK-51

(Maku). Every line was pretested for membrane reactivity with fluorescein-conjugated Mutua and Kipkoech serum before each use. The pretesting procedure was introduced (Klein *et al.*, 1969) because of day-to-day fluctuations in the reactivity of the cells. Lines with 50–70% membrane reactive cells were selected for the blocking test which was performed a few hours later the same day. There was no indication that the various lines differed from each other with regard to the specificity of the reactivity with different conjugates or the blocking patterns obtained. As a rule, each serum was tested against several different lines. A mean blocking index was calculated and used for presentation in the charts. The number of blocking tests performed is indicated for each serum.

Sera

The test sera were obtained from patients with a diagnosis of nasopharyngeal tumour, made in Nairobi and Hong Kong, respectively. Controls included sera derived from healthy African donors (mainly blood relatives of Burkitt lymphoma patients), and sera of Africans with various non-neoplastic or neoplastic diseases other than Burkitt lymphoma and carcinoma of the post-nasal space. Other sera were collected in Calcutta from Indian patients mostly with oropharyngeal carcinomas, which were generously supplied by Dr S. R. S. Rangan and Dr F. Bang, of Johns Hopkins University.

Immunofluorescence test for antibodies to EBV

The procedures for staining of acetone-fixed smears of the EB3 line of Burkitt tumour cells by direct and indirect immunofluorescence and for the titration of antibodies to EBV have been described previously (Henle & Henle, 1966; Henle *et al.*, 1968).

Direct membrane immunofluorescence and blocking test

The fluorescein conjugated reference reagents for direct membrane staining were prepared as described (Goldstein *et al.*, 1969). Two reagents were used, F-Mutua and F-Kipkoech, obtained from two representative sera, derived from the African Burkitt lymphoma patient Mutua Ndinga (Kenya Cancer Council No. 454) and from a 28-year-old African male patient with carcinoma of the post-nasal space, Kipkoech Kituror (KCC No. 883). The clinical pictures of both patients were typical and the diagnoses were histologically confirmed in both. The ability of the reference sera to react with distinctive membrane antigens present on lymphoblastoid cells from EBV carrier lines derived from Burkitt lymphomas and leucocytes of patients with infectious mononucleosis has been described (Klein *et al.*, 1969). For the blocking tests, live, pretested lymphoblastoid cells from EBV carrier lines of Burkitt lymphoma or infectious mononucleosis origins were first incubated with unlabelled test serum and after washing, with F-Mutua or F-Kipkoech. Incubation times, reagent dilutions and processing of the cells were the same as previously reported (Klein *et al.*, 1969). Three controls were included in each test: (a) cells incubated with the conjugate alone; (b) cells incubated with an unconjugated serum known to block the reaction (unconjugated Mutua and/or Kipkoech serum, as a rule); and (c) cells incubated with an unconjugated normal serum, known to lack blocking antibodies for both conjugates (the serum of Berith, a Swedish technician). The blocking activity of each test serum was expressed as the blocking index (BI), calculated by subtracting the proportion of membrane stained cells in the sample exposed to the unlabelled test serum, followed by the conjugate, from the percent-

age of positive cells obtained with the conjugate alone, and dividing the difference by the latter figure.

*Histological examination of tumours**

Through the courtesy of Dr S. McClatchie, Dr J. Itotia, Professor H. Cameron and Dr F. B. Weinstein tumour sections were made available from a proportion of the African cases. These cases included a number of malignant tumours of the post-nasal space other than carcinomas, as indicated.

In the Hong Kong material the patients were selected on the basis of diagnosis; they all had carcinomas. Histological sections were available for re-examination in all these cases.

No sections were available from the Indian tumours.

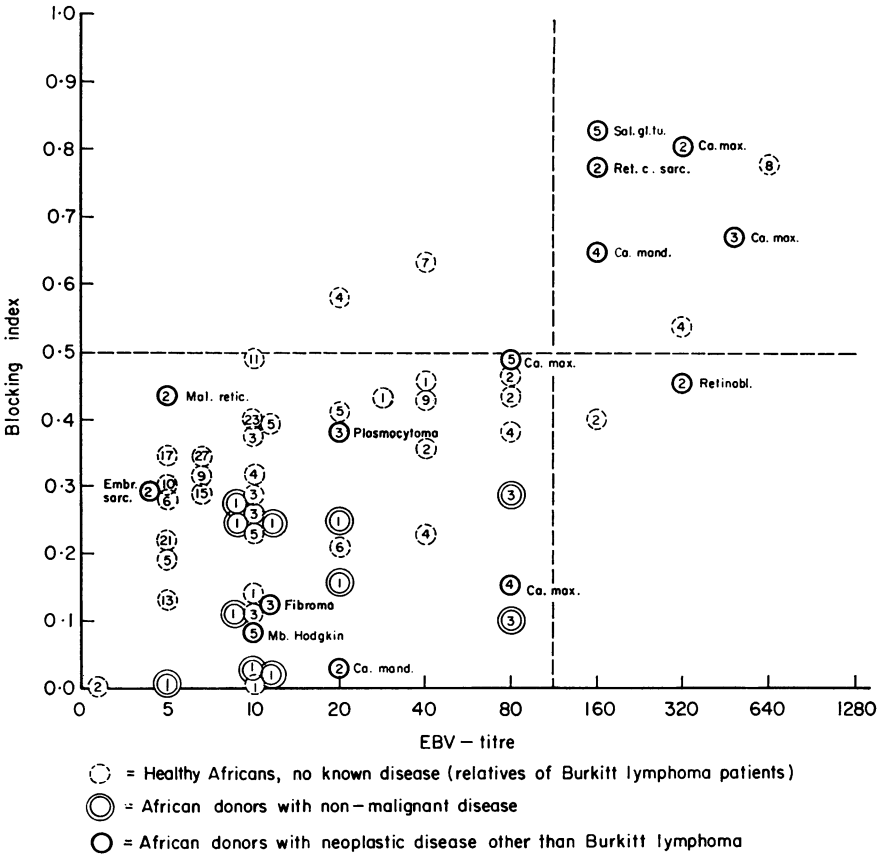

FIG. 1. Sixty-one African control sera tested for blocking against F-Mutua. Blocking indexes shown in relation to anti-EBV titres. The figures within each symbol show the number of blocking tests performed. Distribution of sera in the four quadrants: H_BH_E (seven): high blocking, high EBV; H_BL_E (two): high blocking, low EBV; L_BL_E (fifty): low blocking, low EBV; L_BH_E (two): low blocking, high EBV.

* All tumours were biopsied and histologically examined by the local pathologist. As many sections as possible were collected by the authors and re-examined in order to get a uniform evaluation. Cases where sections were not available are indicated.

RESULTS

African control sera

Sera from sixty-one African donors without known illness or with various non-neoplastic or neoplastic diseases (other than Burkitt lymphoma or nasopharyngeal carcinoma) were tested for blocking activity against F-Mutua. The thirty-six sera from healthy donors were those of blood relatives of Burkitt lymphoma patients, with a mean age of 11 years (range: 1–50). Eleven of the sera were collected from Africans hospitalized for non-malignant diseases with a mean age of 11 years (range 5–25). The remaining fourteen sera were taken from Africans with neoplastic diseases, i.e. four with carcinomas of the maxilla, two with

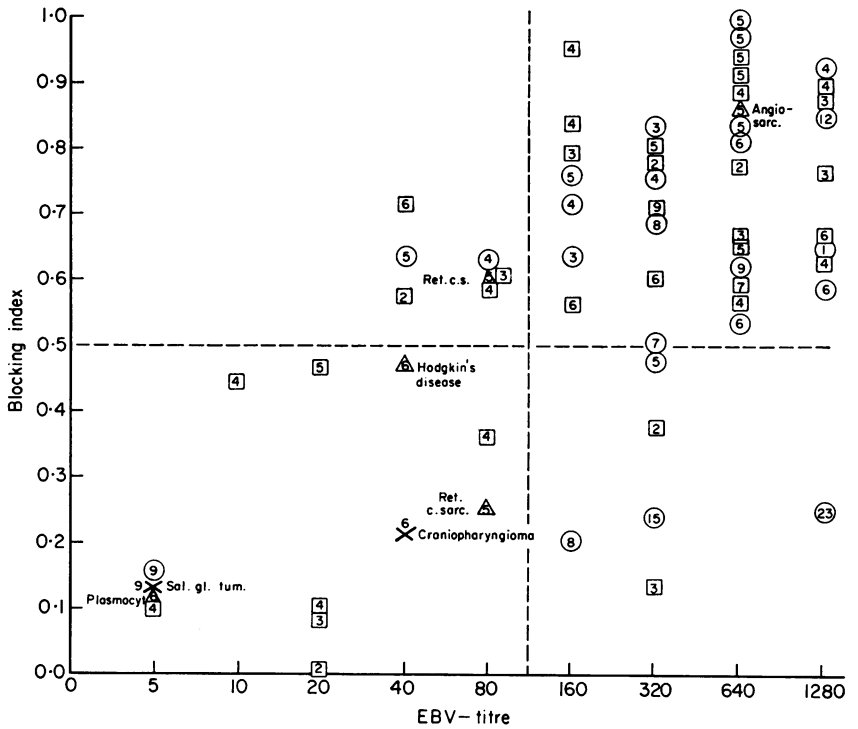

FIG. 2. Sixty-five sera from African patients with nasopharyngeal tumours, tested for blocking against F-Mutua and anti-EBV titre. H_BH_E, thirty-eight, H_BL_E, seven; L_BL_E, thirteen; L_BH_E, seven. The figures within each symbol show the number of blocking tests performed. □, Pathological slides not available; ○, poorly differentiated or anaplastic squamous cell carcinomas (re-examined by the authors); ×, other tumours of epithelial origin (re-examined by the authors); △, sarcomatous tumours (re-examined by the authors).

carcinomas of the lower jaw, one each with a diagnosis of fibroma, salivary gland tumour, plasmocytoma, malignant reticulosis, embryonal sarcoma, reticulum cell sarcoma of the tongue, retinoblastoma and Hodgkin's disease. Their mean age was 30 years (range 5–70). The blocking indices are plotted against the anti-EBV titres in Fig. 1. As before (Klein *et al.*, 1969) a BI of 0·5 was taken as the dividing line between high and low blocking activity. Anti-EBV titres of 1:80 or less were classified as low and titres of 1:160 or more were

considered high. Fifty of the sixty-one African control sera fell in the L_BL_E category (low blocking, low anti-EBV). These included all eleven sera of the non-malignant disease group and eight sera from the neoplastic disease group. The opposite category (H_BH_E) characterized by high blocking and high anti-EBV values, contained seven sera. Five of these were derived from tumour patients, one with a salivary gland tumour, a carcinoma of the mandible and a reticulum cell sarcoma of the tongue, and two with carcinomas of the maxilla. The remaining two sera were collected from relatives of two different Burkitt lymphoma patients; a brother and a grandmother. The sera of two further relatives, a brother and the mother of a third Burkitt tumour patient, fell into the H_BL_E category,

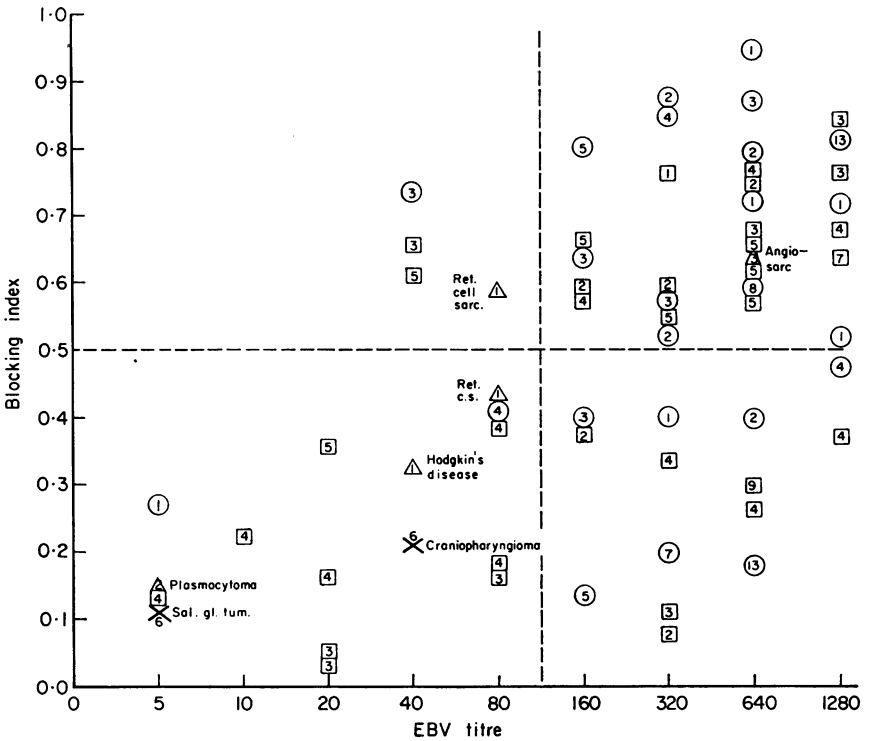

FIG. 3. Sixty-five sera from African patients with nasopharyngeal tumours, tested for blocking against F-Kipkoech and anti-EBV titre. H_BH_E, thirty-one; H_BL_E, four; L_BL_E, sixteen; L_BH_E, fourteen. Key as Fig. 2.

yielding high blocking but low anti-EBV values. The occurrence of sera with membrane reactive antibodies despite very low anti-EBV titres has been noted previously among relatives of Burkitt tumour patients (Pearson *et al.*, 1969).

A selected group of nineteen sera were tested also for their ability to block the F-Kipkoech conjugate. Four sera from donors without known disease and thirteen from donors with neoplastic diseases showed low or insignificant blocking against F-Kipkoech. Only two sera gave high blocking against Kipkoech (they also blocked F-Mutua). These were derived from patients with a mixed salivary gland tumour and melanosis of the nose. Two sera of

patients with carcinomas of the lower jaw and the maxilla, respectively, gave high blocking when tested against F-Mutua, but low blocking of F-Kipkoech. Sixteen of these nineteen sera had low anti-EBV titres.

Sera from African patients with nasopharyngeal tumours

A total of sixty-five sera were available from African patients with the clinical diagnosis of nasopharyngeal tumour. The mean age of the serum donors was 35 years (range: 10–65). The anti-EBV titres and blocking indices against F-Mutua are shown in Fig. 2, which shows

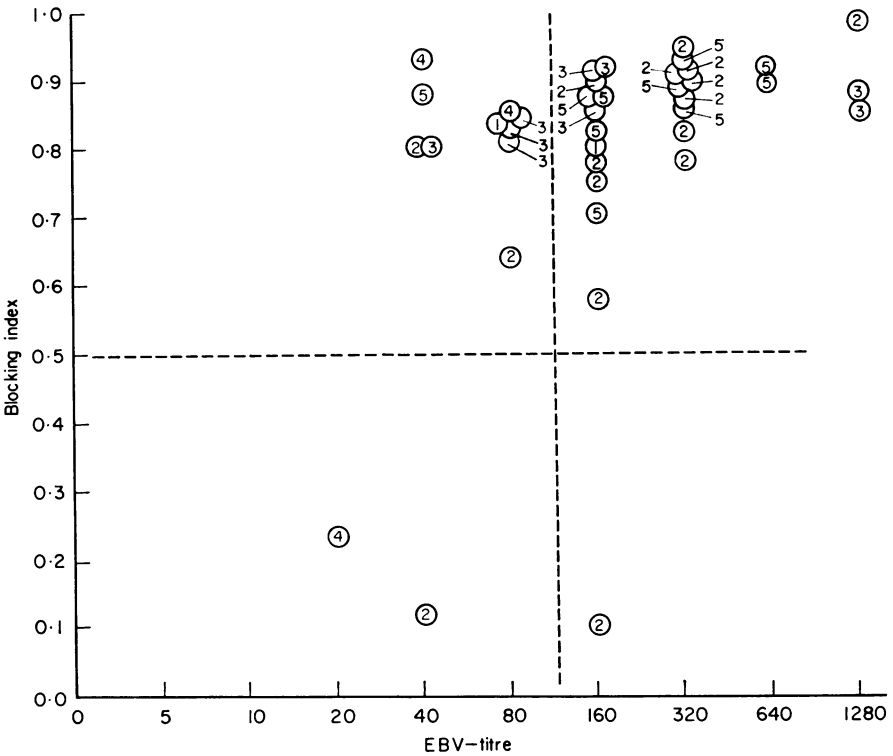

FIG. 4. Blocking ability of forty sera from Chinese donors with nasopharyngeal carcinomas, tested against F-Mutua, shown in relation to anti-EBV titre. H_BH_E, twenty-seven; H_BL_E, ten; L_BL_E, two; L_BH_E, one. ○, Poorly differentiated or anaplastic squamous cell carcinomas (including two cases of moderate high differentiation). All cases re-examined by the authors. The figures within each symbol show the number of blocking tests performed.

that thirty-eight of the sixty-five sera fell into the H_BH_E category. Histological sections were re-examined from seventeen of the thirty-eight tumours. All except one were diagnosed as poorly differentiated or anaplastic squamous cell carcinomas. The exceptional case was classified as an angiosarcoma. The H_BL_E group contained seven sera. Histological sections were available from three of the cases of which two were diagnosed as poorly differentiated or anaplastic squamous cell carcinomas and one as a reticulum cell sarcoma. The L_BH_E group contained seven sera. All five of the histologically re-examined cases were identified as

poorly differentiated carcinomas. Finally, the L_BL_E category comprised thirteen sera. Of the six specimens available for histological re-examination, only one was classified as an anaplastic carcinoma. The remaining five were diagnosed as craniopharyngioma, adenoid cystic carcinoma of salivary gland, plasmocytoma, reticulum cell sarcoma and Hodgkin's disease, respectively.

In summary, of the thirty-one tumours which were histologically re-examined by us, twenty-four were diagnosed as poorly differentiated or anaplastic carcinomas. The sera of all but one of these patients have high anti-EBV and/or high membrane blocking reactivity. In contrast, of the seven patients with tumours that were localized in the nasopharynx but carried other histological diagnoses, five belonged into the doubly low (L_BL_E) group.

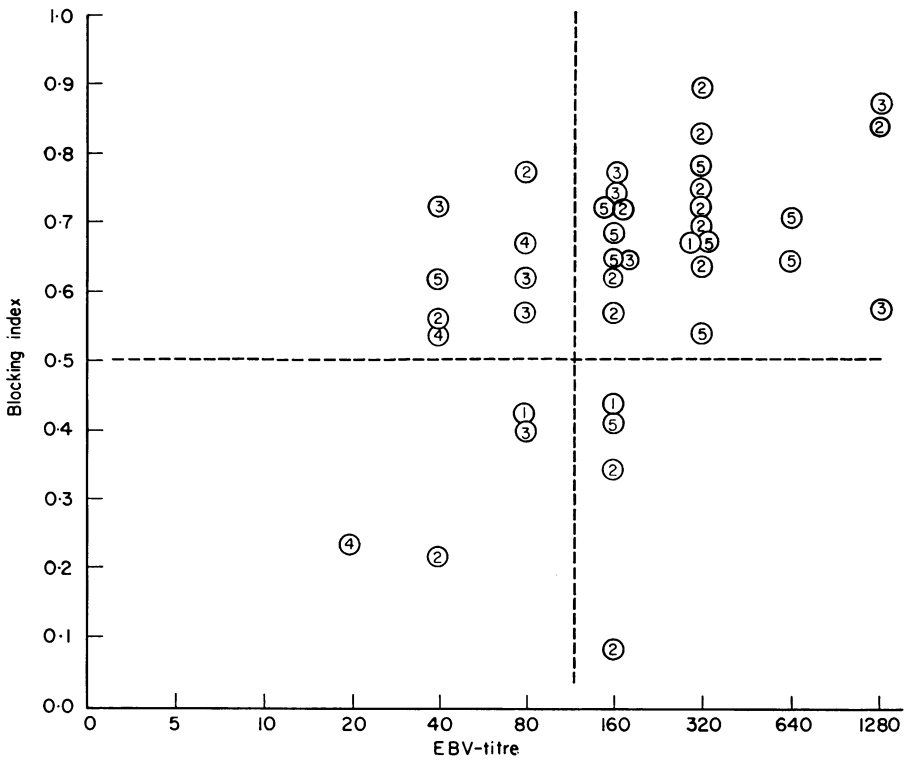

FIG. 5. Blocking ability of forty sera from Chinese donors with nasopharyngeal carcinomas, tested against F-Kipkoech, shown in relation to anti-EBV titre. H_BH_E, twenty-four; H_BL_E, eight; L_BL_E, four; L_BH_E, four. Key as Fig. 4. All cases re-examined by the authors.

The same sixty-five sera from African nasopharyngeal tumour patients were tested also against the F-Kipkoech conjugate. The results are shown in Fig. 3. The blocking patterns against this reagent were essentially similar to those obtained with F-Mutua, but a smaller number of sera blocked F-Kipkoech than F-Mutua; i.e. thirty-one (instead of thirty-eight) of the sixty-five sera were in the H_BH_E and four (instead of seven) in the H_BL_E category. The distribution of histological diagnoses among the doubly low and the singly or doubly high groups remained essentially unchanged.

Sera from Chinese patients with carcinomas of the post-nasal space

A total of forty sera from Chinese donors with CaPNS were available. Their mean age was 47 years (range: 24–70). The results of the blocking tests against F-Mutua are shown in Fig. 4. It is seen that thirty-seven of the forty sera showed high blocking and twenty-seven of these had high anti-EBV titres as well, whereas ten fell into the low anti-EBV category. Of these forty cases thirty-eight were poorly differentiated or anaplastic carcinomas, and two were moderately well differentiated squamous cell carcinomas.

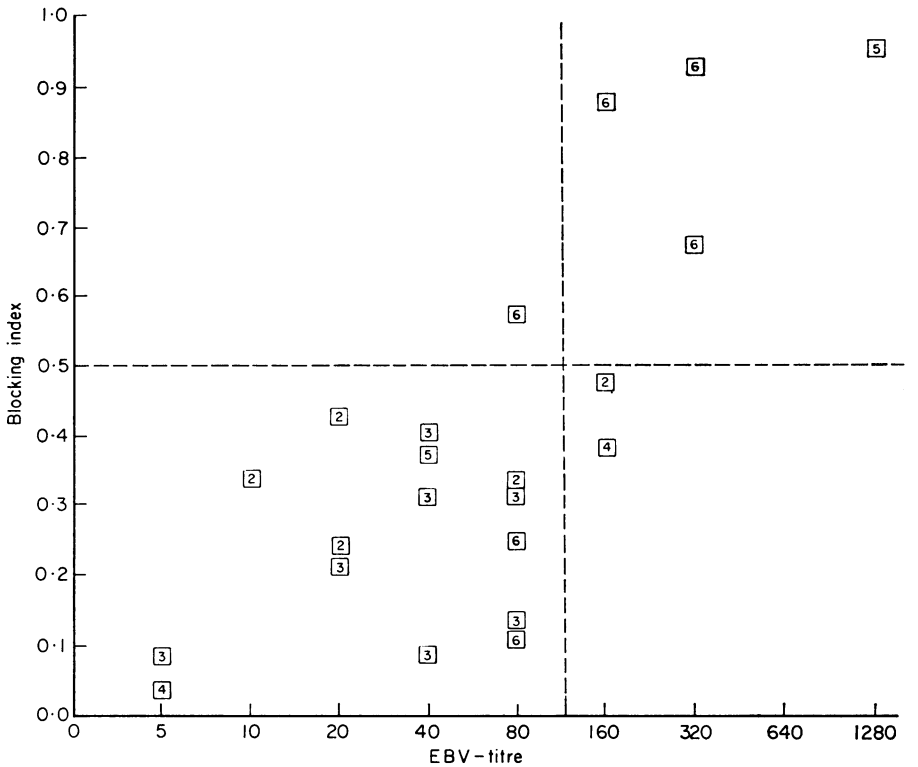

FIG. 6. Blocking ability of twenty-two sera from Indian oropharyngeal carcinoma, etc., cases, tested against F-Mutua, shown in relation to anti-EBV titre. H_BH_E, four; H_BL_E, one, L_BL_E, fifteen; L_BH_E, two. □, Pathological slides not available. The figures within each symbol show the number of blocking tests performed.

The above sera were tested also for their ability to block the F-Kipkoech conjugate. This more stringent test revealed again the remarkable homogeneity of this group; that is, a high incidence of potent blocking sera (Fig. 5). All but five of the thirty-seven sera which showed high blocking when tested against F-Mutua also gave significant blocking of F-Kipkoech.

Sera from Indian patients with buccal, oro- and hypopharyngeal tumours

This group of twenty-two sera was obtained from patients with buccal, oropharyngeal and hypopharyngeal tumours in Calcutta. The mean age of these patients was 53 years (range: 39–74). In Fig. 6 the blocking indices obtained with F-Mutua are plotted against the

anti-EBV titres. The distribution of the sera resembles that of the African control material. The majority, i.e. fifteen of the twenty-two sera, fell into the doubly low (L_BL_E) category. Of these, eight were diagnosed as hypopharyngeal carcinomas, three as carcinomas of the tonsil, two as carcinomas of the base of the tongue, one each as a carcinoma of the soft palate and a salivary adenoma. Two sera gave low blocking despite high anti-EBV titres, both from patients with carcinomas of the soft palate. Only five sera were in the high blocking category. Three of these, showing high anti-EBV titres as well, were derived from patients with squamous cell carcinomas of the hypopharynx, the base of the tongue and the tonsil, respectively. The fourth serum, also with a high anti-EBV titre, came from a patient

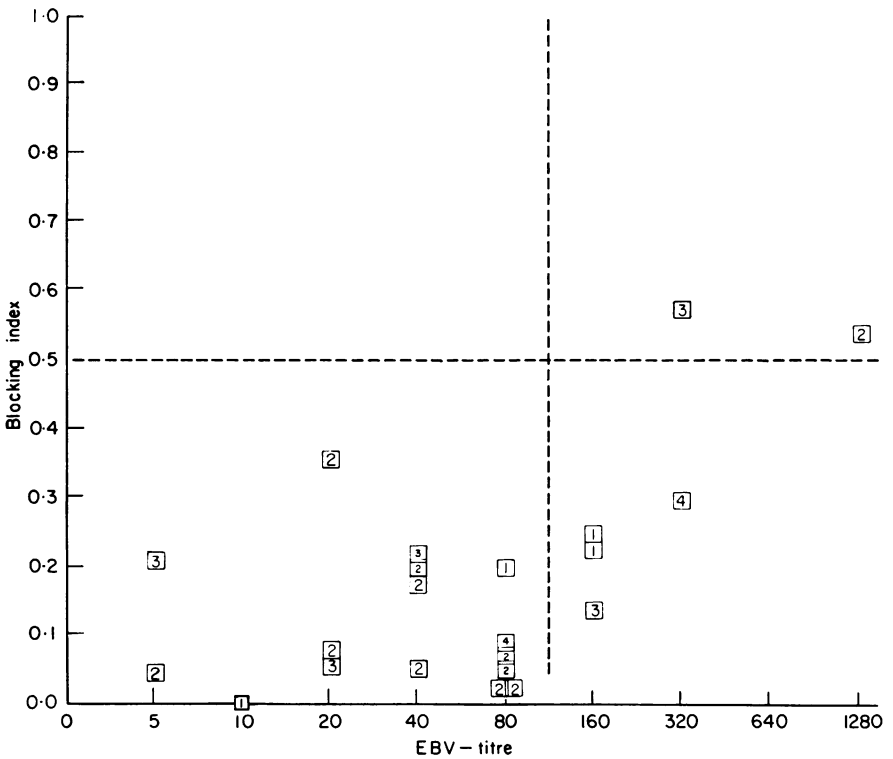

FIG. 7. Blocking ability of twenty-two sera from Indian oropharyngeal cancer, etc., cases, tested against F-Kipkoech, shown in relation to anti-EBV titre. H_BH_E, two; H_BL_E, none; L_BL_E, sixteen; L_BH_E, four. Key as Fig. 6.

with a tumour of the hypopharynx and the fifth with a low anti-EBV level from a case of a squamous cell carcinoma of the tonsil. When tested against F-Kipkoech, all sera fell into the low blocking category (Fig. 7) except two which were derived from patients with squamous cell carcinomas of the base of the tongue and the hypopharynx, respectively.

Comparison of the four groups

Table 1 presents a comparison of the four groups discussed above with regard to age and sex, mean anti-EBV titres, mean blocking values, and, where available, histological classi-

TABLE 1. Sex distribution, age range and mean, average serological values, and distribution of serum reactivity patterns in doner groups tested

Serum donor groups tested	Total No.	Age (mean and range)	Sex			Mean anti-EBV titre (geometric)	Mean BI		No. of cases with high BI		No. of cases with high anti-EBV titre	No. of cases with high BI and/or high anti-EBV titre		No. of cases with *both* high BI *and* high anti-EBV titre		Total No.
			Male	Female	Unknown		F-Mutua	F-Kipkoech	F-Mutua	F-Kipkoech		F-Mutua	F-Kipkoech	F-Mutua	F-Kipkoech	
African controls	61	16 1–70	26	20	15	1:23	0·32	0·28	9/61	2/19	9/61	11/61	3/19	7/61	2/19	
Without known disease	36	11 1–50	16	13	7	1:18	0·33	0·11								
With non-neoplastic disease	11	11 5–25	3	5	3	1:15	0·15	0·28								61
With neoplastic disease other than BL* and CaPNS	14	30 5–70	7	2	5	1:68	0·43	0·39								
African tumours of post-nasal space (thirty-one cases re-examined histologically)	65	35 10–65	39	10	16	1:210	0·59	0·48	45/65	35/65	45/65	52/65	49/65	38/65	31/65	65
Malignant tumours other than carcinomas (all cases re-examined histologically)	7					1:40	0·38	0·35	2/7	2/7	1/7	2/7	2/7	1/7	1/7	
Carcinomas (CaPNS) (all cases re-examined histologically)	24					1:156	0·63	0·58	18/24	15/24	21/24	23/24	22/24	16/24	14/24	
Chinese post-nasal space carcinomas All cases re-examined histologically	40	47 24–70	23	17	0	1:176	0·79	0·62	37/40	32/40	28/40	38/40	36/40	27/40	24/40	40
Indian oro- and hypopharyngeal carcinomas No cases re-examined histologically	22	53 39–74	20	2	0	1:60	0·38	0·18	5/22	2/22	6/22	7/22	6/22	4/22	2/22	22

Serum grouping according to histological diagnosis	Total No.	Mean anti-EBV titre	Mean BI		Percentage of cases with high BI		Percentage of cases with high anti-EBV titre	Percentage of cases with high BI and/or high anti-EBV titre		Percentage of cases with *both* high BI *and* high anti-EBV titre		Total No.
			F-Mutua	F-Kipkoech	F-Mutua	F-Kipkoech		F-Mutua	F-Kipkoech	F-Mutua	F-Kipkoech	
African and Chinese CaPNS Sixty-four cases re-examined histologically	64	1:160	0·73	0·60	86	72	77	95	92	67	59	64
African and Indian oro-pharyngeal tumours other than CaPNS Seven cases re-examined histologically	29	1:53	0·38	0·22	24	14	24	31	27	17	10	29
African controls	61	1:23	0·32	0·28	9	10	15	18	15	11·5	10	61

* BL = Burkitt Lymphoma.

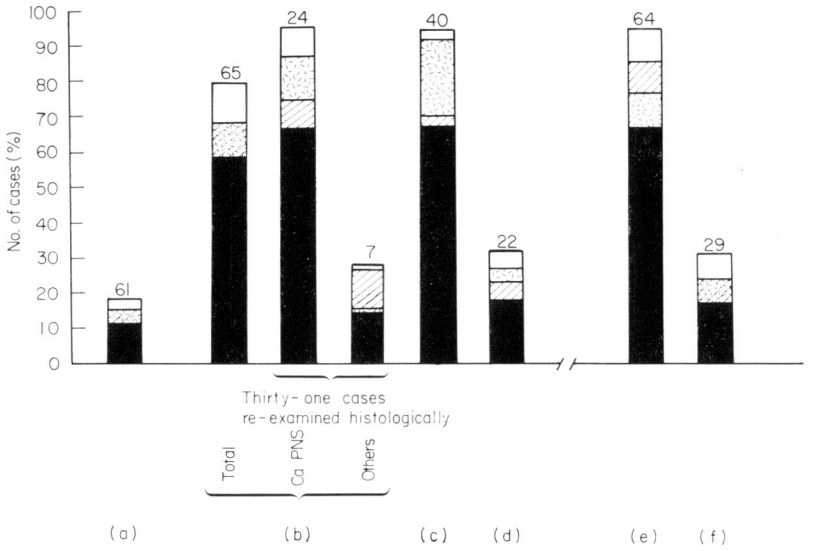

FIG. 8. Comparison of the blocking ability of the sera of donor groups, tested against F-Mutua. Number of cases on top of each column. (a) African controls; (b) African tumours of post-nasal space; (c) Chinese carcinomas of PNS; (d) Indian buccal, oro- and hypopharyngeal carcinomas; (e) all carcinomas of PNS; (f) all post-nasal space tumours other than carcinomas. Open columns, High BI *or* anti-EBV titre; hatched columns, high BI; stippled columns, high anti-EBV titre; solid columns, high BI *and* anti-EBV titre.

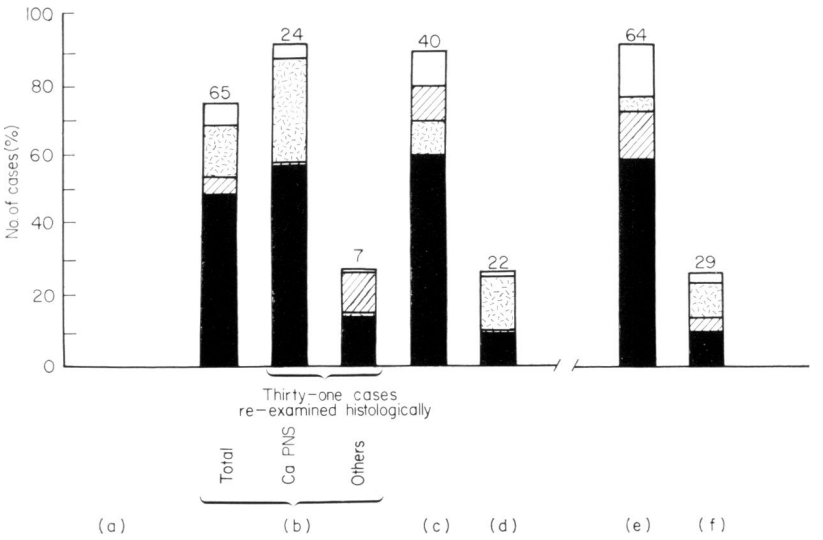

FIG 9. Comparison of the blocking ability of the sera of donor groups, tested against F-Kipkoech. Key as Fig. 8.

fication. The distribution of high and low reactive sera is further analysed in Figs. 8 and 9. It is evident that sera with a high blocking ability against F-Mutua occurred in a high frequency in the African and Chinese patients with CaPNS (75 and 92·5%, respectively). High blocking sera were found less frequently in the African control group, in the African patients with PNS tumours other than carcinomas and amongst the Indian oro- and hypopharyngeal tumour patients (14, 28 and 23%, respectively). The mean anti-EBV titres were also higher in the two groups of nasopharyngeal carcinoma (1:210 and 1:176, respectively) than in the African control or the Indian tumour groups (1:23 and 1:60, respectively). It is interesting to note that although the Hong Kong patients' sera showed a higher and more regular membrane blocking activity against both F-Mutua and F-Kipkoech than the sera from African CaPNS patients, the mean anti-EBV titres were actually somewhat lower in the Chinese than in the African group.

DISCUSSION

In previous studies on the blocking of cell membrane immunofluorescence reaction (Klein *et al.*, 1969; Pearson *et al.*, 1969) a good general correlation was found between the anti-EBV titres and the ability of different sera to block F-Mutua, but the correlation was not absolute; i.e. only about 80% of the sera tested gave concordant results. In the remaining 20% of 'discordant' sera, either high anti-EBV titres and low membrane blocking activity was found or vice versa. This is understandable in light of the fact that different antibodies are responsible for the two reactivities, even though both appear to be determined by the same virus (Pearson *et al.*, 1969).

The fluorescein-conjugated, membrane-reactive antibodies present in the Mutua conjugate interacted with an EBV-determined antigen complex present in the membrane of living lymphoblastoid cells of lines of Burkitt lymphoma or infectious mononucleosis origins. Mutua's serum contained at least two different antibodies capable of reacting with different parts of the cell surface (Pearson *et al.*, 1969). Furthermore, as yet unpublished, cross-blocking experiments showed that Kipkoech's serum contains at least three antibodies against the EBV-determined surface antigen complex. Two of these are identical with the antibodies present in Mutua's serum, whereas the third is distinct. This is manifested by the fact that unconjugated Mutua's serum does not block F-Kipkoech, whereas Kipkoech's serum does block F-Mutua. Thus, all sera which block F-Kipkoech should also block F-Mutua, whereas the reverse should not necessarily be true. Both predictions were fulfilled by the present findings in that sera which blocked F-Kipkoech also blocked F-Mutua, but a number of sera which blocked F-Mutua failed to block F-Kipkoech. Blocking of F-Kipkoech is thus indicative of a more 'complete' spectrum of membrane-reactive antibodies, directed against the EBV-associated surface antigen complex.

In considering the reactivity patterns of all sera examined in the present report, it becomes evident that seventy-six of the total number of 188 sera fell into the high blocking (against F-Mutua)–high anti-EBV category; seven of sixty-one in the African control group, thirty-eight of sixty-five in the African group with nasopharyngeal tumours, twenty-seven of forty in the Chinese group with CaPNS and four of twenty-two in the Indian group with mostly oro- and hypopharyngeal carcinomas. These figures depart strikingly from what would be expected on the basis of a random distribution in that they show a remarkable over-representation of the African and Chinese CaPNS patients in this category, accounting for 86%

of the sera. This lop-sided distribution becomes even more pronounced if high blocking of F-Kipkoech together with high anti-EBV titres are taken as the criterion. Only sixty of 146 tested sera showed high reactivities in both tests of which 92% were derived from the African and Chinese CaPNS patients.

The opposite extreme, low F-Mutua blocking and low anti-EBV titres, was found in eighty of the 188 sera. Of these, fifty occurred in the African control group, thirteen and two among the African and Chinese patients with nasopharyngeal tumours, respectively, and fifteen in the Indian tumour group. Low blocking of F-Kipkoech together with low anti-EBV titres was seen in forty-nine of 146 sera; sixteen of nineteen African controls so tested, sixteen of sixty-five in the African and four of forty in the Chinese groups of CaPNS, and sixteen of twenty-two in the Indian tumour group.

Histopathology of the primary tumour

A high membrane blocking cum high anti-EBV pattern (H_BH_E) has been found most frequently in African and Chinese patients with nasopharyngeal carcinomas. In the Chinese group of forty patients only two had moderately well differentiated squamous cell carcinoma, while the rest had poorly differentiated squamous cell or undifferentiated carcinoma. It is noteworthy that it was not those two with differentiated tumours who had sera belonging to the low reactive category. Nasopharyngeal carcinomas are predominantly of the low differentiation type, and this is particularly true in Chinese patients. The present finding would appear to indicate that a high serological response in the present tests is not necessarily associated with a poor or low differentiation of the carcinoma. It can only be said that with the evidence available a high serological reactivity appears to be associated with only carcinomas in the large majority of cases and not with all types of tumours occurring in the nasopharynx. Although sera from several patients with other tumours, diagnosed as angiosarcoma, salivary gland type tumour, carcinomas of the lower jaw, of the base of the tongue and of the hypopharynx, respectively, also fell into this category, it is particularly noteworthy that this type of reactivity was infrequent among the twenty-two sera from hypo- and oropharyngeal carcinomas collected in Calcutta.

In their studies on the precipitin reaction with a soluble, probably EBV-determined antigen, extracted from cultured Burkitt tumour cells, Old *et al.* (1966, 1968) and Oettgen *et al.* (1967) previously noted a high degree of association between nasopharyngeal carcinoma and precipitating antibodies. Sera from occasional patients with other types of tumours also gave positive reactions, but very much less frequently than sera from patients with CaPNS.

The only other neoplastic disease so far investigated that shows an EBV-associated serological reactivity of comparable regularity and intensity in the same three tests (anti-EBV, membrane blocking and immunoprecipitation), is Burkitt lymphoma (Henle & Henle, 1966; Old *et al.*, 1966, 1968; Klein *et al.*, 1969). In nasopharyngeal carcinoma, as in Burkitt lymphoma, one must now consider the question whether a herpes-type virus is merely carried in the tumour as a passenger, or plays a more profound, causative role. This question cannot be answered with certainty. The passenger hypothesis implies that the neoplastic disease afflicts individuals with or without antibodies to EBV in a random fashion. Since EB virus shows considerable predilection for lymphoid and leukopoietic tissues, neoplastic proliferation of these tissues would increase the antigenic load and thereby the antibody production in anti-EBV-positive, presumably persistently infected individuals. Similar

proliferations, when occurring in anti-EBV-negative individuals, should not change their negative antibody status. At first glance, the African material of nasopharyngeal tumours shows a heterogeneity that appears to be in line with this expectation. Only thirty-eight of the sixty-five sera examined belonged to the highly reactive category in both the anti-EBV and the membrane blocking tests (against F-Mutua); thirteen sera fell into the low reactive group based on both tests; and fourteen sera showed high reactivity in one but not the other test. Although histopathological re-examination could be performed on only thirty-one of the sixty-five cases, the results raise the question whether the serological heterogeneity is not more apparent than real. Five of the six histologically re-examined cases of the low reactive group turned out to be tumours of quite different types than the poorly differentiated epidermoid or anaplastic carcinomas characteristic for the post-nasal space; in fact, most of them were not carcinomas at all. In contrast, sixteen of the seventeen histologically re-examined cases in the high-reactive group (according to both tests) were poorly differentiated or anaplastic carcinomas. The impression that a histopathologically homogeneous group might show a more consistent serological pattern is further reinforced by the findings in the Chinese patients. All forty cases were typical carcinomas of the post-nasal space and all but three belonged to the high blocking group against F-Mutua. Perhaps even more important, thirty-two of the same sera also showed high blocking against F-Kipkoech.

It might be expected that the development of a tumour at a site where lymphoid tissues are abundant, as in the case of nasopharyngeal carcinoma occurring in a part of the Waldeyer's lymphoid ring, would have an associated high serological reactivity, but the low reactivity found in the sera obtained from the Indian donors who had tumours arising from the tonsils and back of tongue where lymphoid tissues are also abundant is not in support of this hypothesis.

The passenger hypothesis would demand a similarly high EBV-associated reactivity in diverse tumours of the lymphoid and reticulo-endothelial system. In our, as yet limited experience, this does not seem to be the case. Sera from a variety of patients with lymphoid tumours, reticulo-endothelioses, reticulum cell sarcomas and leukaemias have shown membrane blocking reactivities within the control range. Their anti-EBV titres covered the range from $<1:10$ to as high as 1:1280 in a few instances. These data (to be published) differ markedly from those obtained with sera from patients with Burkitt tumours or carcinomas of the nasopharynx. Similar findings were obtained with the precipitin test by Oettgen *et al.* (1967).

If one assumes, for the sake of discussion, that a herpes-type virus may play a more important role in the causation of nasopharyngeal carcinomas than a mere passenger, it might act in a number of different ways. Conceivably, it could sensitize the target cell of the neoplastic transformation to the actual carcinogenic agent, thereby increasing the probability of tumour development but without contributing directly to the neoplastic cellular change. As in certain other virus-induced experimental tumour models, it could achieve this, for example, by increasing the number of susceptible target cells. Alternatively, EBV might function in a co-carcinogenic or even carcinogenic capacity. If an oncogenic activity is assumed, it is necessary to postulate the synergistic action of other, environmental carcinogens or co-carcinogens as well. In addition, a genetically determined predisposition may also operate. This could partially explain differences in susceptibility of different groups and individuals, in line with the hypothesis suggested to explain the high risk among Southern Chinese (Ho, 1967b).

It is obviously impossible to decide between these alternatives at the present time. It is of interest that herpes-type virus particles have been described recently in tissue cultures derived from several specimens of nasopharyngeal carcinoma (de-Thé *et al.*, 1969). For further clarification, it seems urgently necessary to establish whether EBV-associated reactivity patterns based on the different available tests, are truly characteristic for a certain histological type or types of carcinomas of the post-nasal space, and not for other malignant tumours of the head and the neck area. Indirect as they are, such serological studies may greatly contribute to the understanding of the aetiology of this tumour. A serological and, possibly, aetiological relationship between carcinoma of the post-nasal space and Burkitt lymphoma may appear very far fetched at this stage. Yet it should be pointed out that the histological origin of nasopharyngeal cancer is still under debate. It has been suggested on histological grounds by at least one author (Weinstein, 1968), that they may arise from thymic remnants. Recent, as yet unpublished experiments by Yata concerning the occurrence of thymus-associated antigens in nasopharyngeal carcinomas would be in line with this suggestion.

ACKNOWLEDGMENTS

This work was supported by Grants CA-04747 and CA-04568, and by Contract PH43-66-477 within the Special Virus Leukemia Program of the National Cancer Institute, National Institutes of Health, United States Public Health Service, the Damon Runyon Memorial Fund for Cancer Research (DRG 598), the Swedish Cancer Society, Lotten Bohman's Foundation, Harald & Greta Jeansson's Foundation and Åke Wiberg's Foundation.

REFERENCES

CLIFFORD, P. (1965) Carcinoma of the nasopharynx in Kenya. *E. Afr. med. J.* **42,** 373.

DE-THÉ, G., AMBROSIONI, J.C., HO, H.C. & KWAN, H.C. (1969) Presence of herpes-type virions in Chinese nasopharyngeal tumor cultured in vitro. *Proc. Amer. Ass. Cancer Res.* **10,** 19.

DIEHL, V., HENLE, G., HENLE, W. & KOHN, G. (1968) Demonstration of a herpes group virus in cultures of peripheral leukocytes from patients with infectious mononucleosis. *J. Virol.* **2,** 663.

GOLDSTEIN, G., KLEIN, G., PEARSON, G. & CLIFFORD, P. (1969) Direct membrane immunofluorescence reaction of Burkitt lymphoma cells in culture. *Cancer Res.*, **29,** 749.

HENLE, G., HENLE, W. & DIEHL, V. (1968) Relation of Burkitt's tumor-associated herpes-type virus to infectious mononucleosis. *Proc. nat. Acad. Sci. (Wash.)* **59,** 94.

HENLE, G. & HENLE, W. (1966) Immunofluorescence in cells derived from Burkitt's lymphoma. *J. Bact.* **91,** 1248.

HINUMA, Y., KOHN, M., YAMAGUDU, J., WUDARSKI, D.J., BLADESLEE, J.R., JR & GRACE, J.T., JR (1967) Immunofluorescence and herpes-type virus particles in the P3HR-1 Burkitt lymphoma cell line. *J. Virol.* **1,** 1045.

HO, H.C. (1967a) *Nasopharyngeal Carcinoma in Hong Kong.* U.I.C.C. Monograph Series 1, 10, p. 58–63.

HO, H.C. (1967b) Panel 11: *Cancer of Nasopharynx*, UICC Monograph Series Vol. 10: Ninth International Cancer Congress (Ed. by R. J. C. Harris) pp. 110–113. Springer-Verlag, Berlin.

KLEIN, G., CLIFFORD, P., KLEIN, E., SMITH, R.T., MINOWADA, J., KOURILSKY, K.M. & BURCHENAL, J.H. (1967) Membrane immunofluorescence reactions of Burkitt lymphoma cells from biopsy specimens and tissue cultures. *J. nat. Cancer Inst.* **39,** 1027.

KLEIN, G., PEARSON, G., HENLE, W., DIEHL, V. & NIEDERMAN, J.C. (1968) Relation between Epstein-Barr viral and cell membrane immunofluorescence in Burkitt tumor cells. II. Comparison of cells and sera from patients with Burkitt's lymphoma and infectious mononucleosis. *J. exp. Med.* **128,** 1021.

KLEIN, G., PEARSON, G., HENLE, G., HENLE, W., GOLDSTEIN, G. & CLIFFORD, P. (1969) Relation between EB viral and cell membrane immunofluorescence of Burkitt tumor cells. III. Comparison of blocking of

direct membrane immunofluorescence and anti-EBV reactivities of different sera. *J. exp. Med.*, **129,** 697.

LEVY, J.A. & HENLE, G. (1966) Indirect immunofluorescence tests with sera from African children and cultured Burkitt lymphoma cells. *J. Bact.* **92,** 275.

MUIR, C.S. (1962) Cancer of the buccal cavity and nasopharynx in Singapore. *Brit. J. Cancer.* **16,** 583.

NADKARNI, J.S., NADKARNI, J.J., CLIFFORD, P., MANOLOV, G., FENYÖ, E.M. & KLEIN, E. (1969) Characteristics of new cell lines derived from Burkitt lymphomas. *Cancer*, **23,** 64.

NIEDERMAN, J.C., MCCOLLUM, R.W., HENLE, G. & HENLE, W. (1968) Infectious mononucleosis. Clinical manifestations in relation to EB virus antibodies. *J. Amer. med. Ass.* **203,** 205.

OLD, L.J., BOYSE, E.A., GEERING, G. & OETTGEN, H.F. (1968) Serologic approaches to the study of cancer in animals and in man. *Cancer Res.* **28,** 1288.

OLD, L.J., BOYSE, E.A., OETTGEN, H.F., DE HARVEN, E., GEERING, G., WILLIAMSON, B. & CLIFFORD, P. (1966) Precipitating antibody in human serum to an antigen present in cultured Burkitt's lymphoma cells. *Proc. nat. Acad. Sci.* (*Wash.*), **56,** 1699.

OETTGEN, H.F., AOKI, T., GEERING, G., BOYSE, E.A. & OLD, L.J. (1967) Definition of an antigenic system associated with Burkitt's lymphoma. *Cancer Res.* **27,** 2532.

PEARSON, G., KLEIN, G., HENLE, G., HENLE, W. & CLIFFORD, P. (1969) Relation between EB viral and cell membrane immunofluorescence in Burkitt tumor cells. IV. Differentiation between antibodies responsible for membrane and viral immunofluorescence. *J. exp. Med.*, **129,** 707.

WEINSTEIN, F.B. (1968) The site of origin of anaplastic tumors of the posterior nasal space. *Cancer in Africa* (Ed. by P. Clifford, C. A. Linsell and G. L. Timms), p. 393. East African Publishing House, Nairobi, Kenya.