# *SEARCH FOR TUMOR-SPECIFIC IMMUNE REACTIONS IN BURKITT LYMPHOMA PATIENTS BY THE MEMBRANE IMMUNOFLUORESCENCE REACTION**

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Virus-induced lymphomas of the mouse contain tumor-specific antigens, as evidenced by the rejection of small numbers of viable neoplastic cells by preimmunized, genetically compatible animals; and the concomitant appearance of antibodies, demonstrated by the cytotoxic reaction *in vitro*, or by the membrane immunofluorescence reaction with living target cells.[1–10]

In the course of previous studies, we have been particularly interested in lymphomas induced by the Moloney virus.[5, 10] When adult mice are inoculated with this agent, they develop high titers of antibodies, capable of reacting with Moloney lymphoma target cells in immunofluorescence and cytotoxic tests, but not cross-reacting with lymphomas induced by the Gross or Graffi viruses.[5, 11, 12]

Mice inoculated with the Moloney agent when newborn often develop tolerance.[13] Virus-neutralizing, cytotoxic, or fluorescent antibodies fail to appear and cannot be induced by a second virus inoculation at adult age.[14] Mice inoculated as newborn develop leukemia earlier and in a higher incidence than mice that received the agent for the first time at adult age. Animals of the latter category show a decrease of their usually high antibody titer when they develop generalized leukemia. If leukemia is inhibited by chemotherapy, the antibody levels may rise again.[15]

Whether similar reactions occur in human malignancies of the lymphatic system is of interest. The Burkitt lymphoma has attracted much interest during recent years due to serious consideration of a possible virus etiology. All virus-induced murine neoplasms studied have been found to contain tumor-specific antigens capable of inducing host rejection reactions, with common specificity shared by all neoplasms induced by the same virus. The possible antigenicity of the Burkitt tumor is also indicated by the total regression of the disease after chemotherapy in a considerable proportion of cases and the absence of recurrence during observation periods up to 4–6 years. Such durable remissions have been obtained by different agents and even in cases where treatment was incomplete.

According to Burkitt,[16] these good results cannot be attributed to an unusual susceptibility of the tumor to cytotoxic agents since this cannot explain spontaneous or temporary remissions following serum transfusions alone.[24] It has therefore been postulated that some immunological mechanism operates.

The purpose of the present study was to look for possible tumor-specific reactions in Burkitt lymphoma and, if found, to assess their significance. The choice of materials and methods was based on our experience in the Moloney system. Since different Moloney lymphomas showed great variation with regard to surface antigen concentration, and since variants with a decreased antigen concentration could be selected during passage *in vitro* or *in vivo*, we have worked with fresh biopsy material rather than with established cell lines. The membrane immunofluorescence

reaction, based on the use of living target cells,[17] was the method of choice since in our experience this was the most sensitive reaction for the detection of humoral antibodies directed against tumor-specific antigens in Moloney lymphomas.[5, 10]

*Materials and Methods.*—Fresh biopsy specimens were taken from Burkitt tumors in Nairobi and shipped to Stockholm immediately by the most direct air route (SAS), as a rule reaching the laboratory within 24 hr after the operation. During shipment, the sterile specimens were immersed in Eagle's medium and packed in wet ice within thermos bottles. All sera were shipped frozen in dry ice. Upon arrival, parts of the biopsy specimens were finely minced in tissue culture medium and gently shaken by hand for a few minutes. A large number of free cells went readily into suspension, the majority larger than the normal lymphocytes and highly uniform in size. Coulter counter (model B) assays of cell size were regularly performed indicating that 40–60% of the cells corresponded to a large lymphoblast type, clearly distinguishable from the small lymphocytes. Occasional necrotic samples containing less than 70% viable cells were discarded.

All human sera were inactivated at 56°C for 45 min and stored at −20°C.

The indirect (sandwich) fluorescent antibody technique was used as applied by Möller[17] to demonstrate H-2 isoantigens on the surface of *living* mouse cells, and later adapted to tumor-specific Moloney lymphoma antigens.[5] Two million cells were suspended in 0.01 ml basal salt solution (BSS), added to 0.05–0.1 ml undiluted human serum, and incubated for 20 min at 37°C. Sixteen tubes containing aliquots of the same target cell suspension but incubated with different sera were processed in parallel. Between 6 and 20 series were run within 24 hr after the arrival of each biopsy specimen. The reactions observed tended to weaken considerably when the cells were stored beyond 48 hr in Eagle's medium at +4°C.

After incubation, the cells were washed twice and incubated for 20 min at 37°C with 0.05 ml 1:10–1:20 diluted, fluorescein isothiocyanate conjugated rabbit antihuman globulin. After two washings with BSS, 1 drop of 50% glycerol was added to the sediment, as suggested by Lejneva *et al.*[18] All tubes were designated by code and read as blind samples. The proportion of positive cells (for criteria see ref. 5) was estimated by counting 50–100 cells and calculating the fluorescence index as previously described for the Moloney system.[5] For the final evaluation, only reactions giving an F.I. $\geqq$ 0.5 (i.e., at least 50% of the cells showing no membrane fluorescence in the control sample had to be positive in the test sample) were evaluated as positives. This is admittedly rather conservative, but it seemed preferable to underestimate the reactions and risk false negatives, rather than to err on the positive side. An effort was also made to assess the agglutination and fluorescent staining of the red cells included in the target cell suspension.

Table 1 lists the numbers of different sera and target cells that have been tested.

All "Burkitt serum donors" were histologically confirmed Burkitt lymphoma patients. More details concerning them are being published elsewhere.[19] All inpatients, with or without malignant diseases, were from the Kenyatta National Hospital. "Non-Burkitt target cell donors" were Swedish inpatients at the Karolinska Hospital in Stockholm. This category includes lymph node cells from a 51-year-old man with lymphatic leukemia, from a 57-year-old woman with suspected lymphoma, and from a 12-year-old boy with Hans-Schüller-Christian disease. Bone marrow cells were taken from a 57-year-old man with chronic myeloid leukemia and from some of the Burkitt target cell donors themselves (cf. *Results*). Since there was no difference between the results obtained with these four cell types, they have been pooled and are presented together.

*Results.*—(a) *Sera from Burkitt lymphoma patients:* Figure 1 summarizes the reactions obtained with all Burkitt sera against all Burkitt and non-Burkitt target cells (except the Burkitt patients' own bone marrow cells, shown separately). The category of chemotherapeutic response is indicated. Detailed clinical data have been given in another paper,[19] where each patient is identified by the same Kenya Cancer Council number (K.C.C.). Category 1 corresponds to total regression of the tumor after chemotherapy and the disease not being evident elsewhere in the body; 2 indicates marked response but not total regression after at least one course of therapy; 3 indicates moderate response to at least one course of therapy; 4 indicates no response to therapy.

TABLE 1

SUMMARY OF MATERIALS TESTED

| Number | Age group | Total | ≧3 Tests performed |
|---|---|---|---|
| Sera | ... | 171 | ... |
| Serum donors | ... | 90 | ... |
| Burkitt serum donors | 2–15 | 24 | 17 |
| Inpatient serum donors with no malignant diseases | 6–50 | 11 | 9 |
| Inpatient serum donors with malignant diseases other than Burkitt | 2–50 | 27 | 17 |
| Healthy relatives of Burkitt patients | 5–16 | 16 | 9 |
| Swedish healthy blood donors | * | 12 | 12 |
| Number | | | |
| Burkitt target cell donors | ... | 14 | ... |
| Biopsies (1–3 per patient) | ... | 27 | ... |
| Autochthonous combinations tested | ... | 12 | ... |
| Non-Burkitt target cell donors | ... | 4 | |

* Young adults; detailed information on age not available.

It will be seen from Figure 1 that the sera of some patients gave positive reactions against Burkitt target cells in half or more of all tests, whereas others gave lower proportions of positives and some were mainly negative. None of the positive sera tested showed any reactivity against non-Burkitt target cells, but the number of such tests is still rather small.

Two main questions arise. One is whether the reactions were tumor-specific or could be explained in some other way, e.g., as being due to isoantibodies directed against blood group or transplantation antigens. The second question concerns the possible relationship between the reactions and the clinical course of the disease, particularly as regards long-term remission after chemotherapy.

Figure 1 is a pool of all reactions seen with sera from Burkitt donors. Details on the number of biopsy specimens tested and the change in serum reactivities in the course of the disease are given in a detailed paper.[22] It should be mentioned that a positive serum tended to give positive reactions against *all* Burkitt target cells tested (between two and ten donors), but not against non-Burkitt cells. This suggests that the reaction may be directed against some antigen common for Burkitt cells. More conclusive evidence can be obtained, and isoantibody reactions against blood group antigens, transplantation antigens, etc., can be excluded by testing the sera against the donor patient's own, autochthonous tumor cells. This was done in some cases, and Table 2 shows the results in comparison with tests performed against allogeneic Burkitt and non-Burkitt target cells.

The percentage of positive tests was of the same order or higher with autochthonous than with allogeneic tumor cells in five of six cases and in only one case (Mut. 454) was it lower. However, this tumor went eventually to complete regression after chemotherapy. The two biopsy samples, obtained at about this time, were brought into suspension with difficulty. The cells were variable in size and shape, probably including many nontumor cells and degenerating tumor cells.

The results summarized in Figure 1 suggested a certain relationship between the response to chemotherapy and the frequency of positive tests. Patients with more durable regression seemed to show a more consistent antibody response than patients in whom therapy had little or only temporary effect. The clinical histories of the more extensively tested patients were therefore compared with their reactivity pattern in the immunofluorescence tests. The over-all evaluation is shown in Table 3 (for more details see ref. 20). The patients are grouped according to the category of response. Highly significant differences were obtained between the

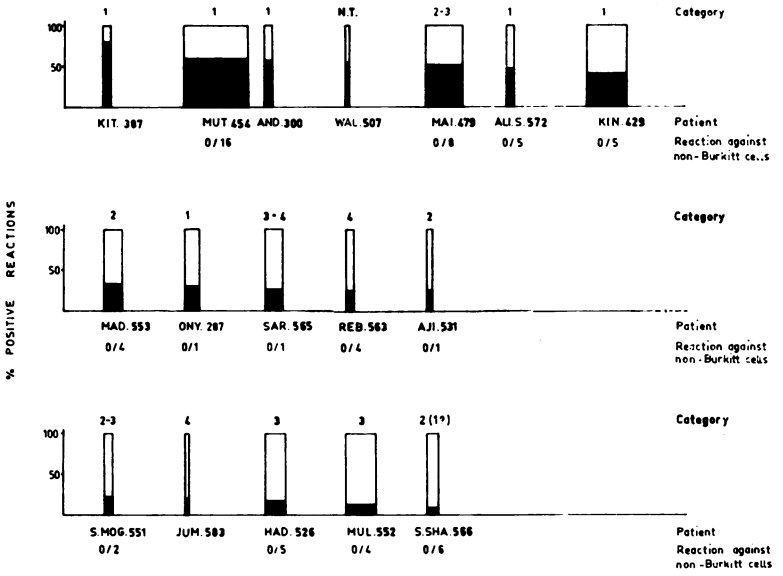


FIG. 1.—Graphical illustration of test results with sera of histologically confirmed Burkitt lymphoma patients, tested against Burkitt lymphoma target cells from 14 different donors. Each column corresponds to one serum donor. Data obtained with serum samples collected at different times have been pooled for each donor. The shadowed portion of each column corresponds to the proportion of positive reactions. The columns are arranged in decreasing order of positivity. The category indicated at the head of each column indicates the chemotherapeutic response (see text). The patients are identified by abbreviated name and the K.C.C. number. The reactions carried out with non-Burkitt target cells are shown under the corresponding name. The width of each column indicates the number of tests performed. The smallest unit corresponds to at least 6 and not more than 10 tests. Above 10, each added unit of the same width corresponds to 10 additional tests. *N.T.*, not tested.

positive reactions seen in categories 1 (108/183, 59%) and 2 (106/278, 38%, $\chi = 19.3$, $P < 0.001$); between categories 1 and 3 (42/210, 20%, $\chi = 63.1$, $P < 0.001$), and between categories 2 and 3 as well ($\chi = 18.6$, $P < 0.001$). Category 4 comprised only two cases, and was not included in the statistical evaluation. In the case of the patients where the chemotherapeutic response changed in the course of the treatment, there was also a change in the percentage of positive tests which appeared to be in line with the change in therapeutic response.

Erythrocyte fluorescence and agglutination were also scored in the Burkitt cell

TABLE 2

REACTIVITY OF BURKITT PATIENT SERA AGAINST ALLOGENEIC AND AUTOCHTHONOUS BURKITT TARGET CELLS, AND AGAINST NON-BURKITT TARGET CELLS

| Serum donor patient (K.C.C. no.) | Number of serum specimens tested | Positive/Total against All Burkitt Target Cells Tested | Per cent | Positive/Total against Allogeneic Burkitt Cells Only | Per cent | Positive/Total against Autochthonous Burkitt Cells | Per cent | Positive/total against allogeneic non-Burkitt cells |
|---|---|---|---|---|---|---|---|---|
| Had. (526) | 3 | 9/54 | 17 | 9/49 | 18 | 0/5 | ... | 0/5 |
| Jum. (583) | 2 | 2/10 | 20 | 2/5 | ... | 0/5 | ... | ... |
| Mad. (553) | 4 | 15/45 | 33 | 6/26 | 23 | 9/19 | 47 | 0/4 |
| Mai. (479) | 11 | 49/96 | 51 | 33/67 | 50 | 16/29 | 55 | 0/7 |
| Mul. (552) | 10 | 11/78 | 14 | 9/63 | 14 | 2/15 | 15 | 0/4 |
| Mut. (454) | 12 | 94/161 | 58 | 87/135 | 64 | 7/26 | 27 | 0/16 |
| Reb. (563) | 3 | 4/16 | 25 | 3/10 | ... | 1/6 | ... | 0/4 |
| S.Mog. (551) | 3 | 5/22 | 23 | 5/21 | 24 | 0/1 | ... | 0/2 |
| S.Sha. (566) | 4 | 3/30 | 10 | 2/19 | 11 | 1/11 | 9 | 0/6 |
| Sar. (565) | 8 | 12/46 | 26 | 9/40 | 23 | 3/6 | 50 | 0/1 |

TABLE 3

SUMMARY OF THE RELATIONSHIP BETWEEN RESPONSE TO CHEMOTHERAPY AND SEROLOGICAL REACTIVITY AGAINST BURKITT TARGET CELLS

| Category | K.C.C. no. | Name | | No. positive tests/total no. of tests | Positive tests, % |
|---|---|---|---|---|---|
| 1* | 572 | Ali | | 8/17 | 47 |
| 1 | 300 | And. | | 11/13 | 85 |
| 1 | 387 | Kit. | early | 18/19 | 95 |
| | | | late | 2/6 | |
| 1 | 287 | Ony. | | 10/32 | 31 |
| 2 | 429 | Kin. | early | 17/54 | 31 |
| 1 | | | late | 17/27 | 62 |
| 2 | 454 | Mut. | early | 29/70 | 41 |
| 1 | | | late | 42/69 | 60 |
| 2 | 437 | Tal. | | 1/4 | ... |
| 2 | 553 | Mad. | | 15/45 | 33 |
| 2 | 531 | Aji. | | 3/12 | 25 |
| 2–3 | 551 | S. Mog. | | 5/22 | 23 |
| 2 | 479 | Mai. | early | 36/70 | 51 |
| 3 | | | late | 10/28 | 35 |
| 3 | 526 | Had. | | 9/54 | 17 |
| 3 | 552 | Mul. | | 11/78 | 14 |
| 3–4 | 494 | Rex. | | 0/4 | ... |
| 4 | 583 | Juma | | 2/10 | 20 |
| 3–4 | 565 | Sar. | | 12/46 | 26 |
| 4 | 563 | Reb. | | 4/16 | 25 |

MEANS AND CHI SQUARE TESTS

| Category | No. of patients | Total positive/ total tests | Per cent positive |
|---|---|---|---|
| 1 | 7 | 108/183 | 59 |
| 2† | 7 | 106/278 | 38 |
| 3‡ | 5 | 42/210 | 20 |
| 4 | 2 | 6/26 | 23 |

Chi square test: between 1 and 2: $\chi^2 = 19.3$, $P < 0.001$; between 1 and 3: $\chi^2 = 63.1$, $P < 0.001$; between 2 and 3: $\chi^2 = 18.6$, $P < 0.001$.

* Categories of chemotherapeutic response:[19, 20] 1, total regression; 2, marked but not total regression to at least one course of therapy; 3, moderate regression to at least one course of therapy; 4, no response to any form of therapy.
† Includes 2–3.
‡ Includes 3–4.

TABLE 4

RED CELL AND BURKITT TARGET CELL REACTIVITY FROM SAME DONOR AFTER EXPOSURE TO SAME BURKITT SERA

| Serum donor | Target Cell Donor: Reb. 563 R* | Reb. 563 L* | S.Sha. 566 R | S.Sha. 566 L | Waf. 584 R | Waf. 584 L |
|---|---|---|---|---|---|---|
| 287 Ony. | + | − | − | − | − | − |
| 572 Ali | + | + | − | − | NT† | NT |
| 566 S.Sha. | NT | − | − | − | NT | − |
| 563 Reb. | − | − | NT | NT | − | − |
| 584 Waf. | + | − | NT | NT | − | − |
| 551 S.Mog. | NT | NT | NT | NT | − | + |
| 454 Mut. | − | +++ | − | +++ | − | +++ |
| 526 Had. | − | − | NT | ± | NT | − |
| 565 Sar. | + | − | − | − | − | − |
| 552 Mul. | − | − | − | − | − | − |
| 429 Kin. | − | + | − | ++ | − | + |
| 479 Mai. | − | +++ | NT | NT | NT | NT |

* *R*, red cell reactions; *L*, Burkitt lymphoma cell reactions.
† NT, not tested.

suspensions. There was no correlation between the red cell and Burkitt cell reactivity in a given serum-cell donor combination (Table 4). The patterns obtained are given in detail elsewhere.[20] In some cases, red cells were positive, whereas Burkitt cells were negative, indicating that some isoantibodies may be present without giving a discernible fluorescence against Burkitt cells. The opposite was also true; some of the most regularly Burkitt-positive sera (such as 454 Mut., 429 Kin., and 479 Mai.) failed to react against erythrocytes of the same Burkitt donor.

It cannot be excluded that there may be other isoantibodies which do not show visible erythrocyte reactions, but nevertheless affect the Burkitt target cells. The only means of checking this is by testing the autochthonous combination whenever possible. In cases where this is not possible, efforts are being made to test the tumor cells in parallel with normal cells from the same patient. One such test is exemplified in Table 5, showing the reactivity of normal bone marrow cells and Burkitt tumor cells from the same patient (Jum. 583) against some selected sera. While the Burkitt cells showed regular reactivity, no significant reactions could be registered with bone marrow cells.

(b) *Sera from non-Burkitt donors:* Twelve sera were tested from young, healthy Swedish

blood donors. Each serum was tested against at least three different Burkitt cells and eight were tested against more than five. No positive results were obtained. These sera were routinely included in later tests as negative controls.

Eleven sera were tested from African inpatients with nonmalignant diseases; nine showed some reactivity (10–50% positive tests[20]); two were negative.

Sera were also tested from 27 African inpatients with malignant diseases other than Burkitt lymphoma. Eleven gave positive reactions varying between 10 and 50 per cent. Sixteen were negative. One of the positive sera was also positive against one non-Burkitt target cell sample.[20]

Sera were also collected from healthy blood relatives, in most cases brothers and sisters of Burkitt patients with histologically confirmed diagnosis, and, as a rule, participating in this investigation themselves. Two of 16 gave a low incidence of positive reactions ($^1/_3$ and $^2/_8$, respectively) while the rest were negative.

TABLE 5

REACTIVITY OF BURKITT LYMPHOMA CELLS AND NORMAL BONE MARROW CELLS FROM THE SAME DONOR (JUM. 583, BLOOD GR. O RH+) EXPOSED TO REPRESENTATIVE, POSITIVE BURKITT SERA

| Serum donor | Test | Fluorescence Index (F.I.)* against Tumor cells | Fluorescence Index (F.I.)* against Bone marrow cells |
|---|---|---|---|
| Reb. 563 | a | 0.32 | 0 |
| | b | 0.66 | 0.14 |
| | c | 0.45 | 0 |
| Mut. 454 | a | 0.45 | 0 |
| | b | 0.43 | 0.24 |
| Wal. 507 | a | 0.39 | 0 |
| | b | 0.90 | 0 |
| Mul. 552 | a | 0.31 | 0 |
| | b | 0.77 | 0 |
| | c | 0.43 | 0 |
| Mut. 454 | a | 0.66 | 0.19 |
| | b | 0.90 | 0 |

* Calculated as the proportion of negative cells in the sample exposed to a standard control serum, minus the proportion of negative cells in the sample exposed to the test serum, divided by the former figure.

*Discussion.*—The data presented in this paper represent a first attempt to study tumor-specific antigenicity in the Burkitt lymphoma. The data are preliminary. Three main points may be considered: (1) Does the reaction detect antibodies specifically directed against Burkitt lymphoma cells? (2) Is there any relationship between the reactions and the clinical course, particularly prolonged regression after chemotherapy? (3) Do the reaction patterns obtained with different serum donors give any information concerning the etiology and epidemiology of Burkitt's disease?

(1) Concerning the first question, while a tumor-specific reaction cannot be regarded as definitely established, several findings support this possibility. In five of six cases where reactive sera could be tested against autochthonous Burkitt target cells, the incidence of positive reactions was of the same order as obtained with the same sera against allogeneic Burkitt cells. Positive sera reacting with one type of Burkitt cell tended to react with all or most other Burkitt cells tested as well. They were regularly negative against cells of other origin. Admittedly, the number of non-Burkitt cells tested is still very small (four), but the complete absence of reactions in all cases except one (with a non-Burkitt serum) strongly supports the argument. There was no correlation between the reactivity of positive sera against allogeneic Burkitt tumor cells, and their activity against the erythrocytes of the same donor. In the cases where positive sera could be tested against bone marrow from the same Burkitt patient, no reactions were seen.

(2) With regard to a possible relationship between prolonged remission after chemotherapy and an immunological reaction, such synergistic action has been demonstrated in experimental tumors with regard to chemotherapy and radio-

therapy,[21, 22] and the curability of choriocarcinoma by chemotherapy has been attributed to a presumed antigenicity of neoplastic cells of fetal origin. We have some evidence that an immunosensitive Moloney mouse lymphoma is more easily influenced by methotrexate than an immunoresistant lymphoma of the same origin.[23]

The prolonged and possibly total regression of the Burkitt lymphoma in a number of patients treated with chemotherapy[16, 19, 24] has also been regarded as possibly due to the combined effect of tumor cell sensitivity to the drug and the host immune response. This is supported by our finding of a correlation between the serological reactivity of Burkitt patients against Burkitt cells in the membrane immunofluorescence test and the durability of their response to chemotherapy.

(3) The possible epidemiological aspects of the present study are even more preliminary. It has been surmised that the Burkitt lymphoma may be an insect-borne virus disease with variable expression, depending on the immunity status. The fact that a certain number of positive sera were found in other categories, including hospitalized African patients with or without malignant diseases and healthy relatives of the Burkitt patients, would fit this picture. On the other hand, since the reactivity of different Burkitt target cells was more variable against the positive non-Burkitt sera than against Burkitt sera, the specificity of this reaction appears more uncertain and the question is left open awaiting evidence.

*Summary.*—As a first approach toward a study of possible tumor-specific antigenicity in Burkitt lymphoma, the sera of Burkitt patients in various phases of the disease, untreated and treated by chemotherapy, were tested against allogeneic and autochthonous Burkitt lymphoma cells derived from fresh biopsy material. Normal lymph node cells or leukemic cells of Swedish patients were used as controls. Further controls included the erythrocytes of the Burkitt lymphoma donor patients themselves and, in a few cases, their normal bone marrow cells.

The sera were tested by the indirect membrane immunofluorescence reaction against living cells. In experimental systems this method was particularly suitable for the demonstration of transplantation antigens or tumor-specific antigens on lymphoma cells. The sera of certain patients gave a high incidence of positive reactions against Burkitt cells; other sera were less frequently positive and still others were negative. The Burkitt positive sera showed no reactivity against the non-Burkitt cells so far tested. A highly significant correlation was found between the durability of the response to chemotherapy and the frequency of positive immunofluorescence tests against Burkitt target cells. In three cases, when the responsiveness of the patient to chemotherapy changed in the course of the disease, there seemed to be a parallel change in serum reactivity.

While the specificity of the reaction cannot be regarded as conclusively established, its possibly tumor-specific nature is suggested by the similar frequency of positive reactions obtained with autochthonous and allogeneic Burkitt cells in five of six cases where such a comparison was possible; by the tendency of positive sera to react with all or most Burkitt cells tested, taken from five to nine patients in most cases; by the absence of reactivity against allogeneic non-Burkitt cells; by the absence of any relationship between erythrocyte agglutination and immunofluorescence and Burkitt cell fluorescence in cases where both were derived from the same donor; and by the absence of reactivity against normal bone marrow cells from the Burkitt cell donor upon testing against the same, Burkitt positive sera.

Twelve sera of healthy Swedish blood donors gave negative results when tested against Burkitt target cells. A small number of positive reactions were obtained in each of the following serum donor categories: hospitalized African patients with nonmalignant diseases, hospitalized African patients with malignant diseases other than Burkitt's lymphoma, and healthy blood relatives of the Burkitt patients.

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