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Surface IgM-kappa Specificity on a Burkitt Lymphoma Cell *In Vivo* and in Derived Culture Lines[1]

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SUMMARY

An exceptional Burkitt lymphoma patient, a 16-year-old boy, yielded tumor biopsy cells and derived tissue culture lines that displayed a strong surface accumulation of IgM heavy and kappa light chain specificities judged by direct membrane fluorescence and cytotoxicity tests. This property was maintained unchanged in the course of more than 5 months of serial passage *in vitro*. A fourth biopsy, obtained from the patient after massive necrosis had been induced in the tumor by cytosine arabinoside chemotherapy, did not show this property at either the biopsy stage or in the derived cell line. The possibility that the neoplastic transformation of lymphoid cells may have afflicted a cell specialized to carry immunoglobulin on its surface may be considered. The phenomenon has to be distinguished from immunoglobulin coating *in vivo* seen with certain biopsy samples from this and other patients. The latter type of coating can be of IgM, IgG, or IgA nature and disappears rapidly on cultivation *in vitro*.

INTRODUCTION

The paper presented at the Symposium consisted of two parts. The first part dealt with the interaction of Burkitt biopsy and tissue culture cells with human sera of various types, studied by indirect membrane immunofluorescence tests. The second part described the accidental finding of an exceptional Burkitt lymphoma cell, showing surface accumulation of IgM and kappa chain both in biopsies and in derived tissue culture lines detected by direct membrane fluorescence and cytotoxic tests. The first part of this study has been summarized recently in another article in this journal, as part of another symposium (10). The second part of the paper has not been published in detail; only a brief preliminary note has appeared recently (6). The present paper is therefore entirely restricted to the second part; concerning the antigenic analysis of Burkitt cells by the indirect test, reference is made to the other symposium paper (10).

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[2] Fellows of the Swedish International Development Authority (SIDA) on leave from Indian Cancer Research Centre, Bombay.

MATERIALS AND METHODS

Origin and Characterization of the Daudi Cell Lines

Case History. Daudi Onyango, 16-year-old African (Luo) male, Kenya Cancer Council (KCC) number 750, was admitted to Nyaza Provincial Hospital, Kisumu, on December 8, 1966, with tumors of the right occiput, left orbita, left abdomen, and right tibia. Biopsy of the left orbita was reported as Burkitt lymphoma. He was treated with 50-mg doses of Cytoxan (2H-1, 3,2-oxazophospharine,2[bis(2-chloroethyl) amino]tetrahydro-), administered orally three times daily for 10 days (total dose, 45 mg/kg), which appeared to produce total tumor regression. The left eye which had been destroyed as a result of the original tumor growth was enucleated on January 25, 1967, after which the patient was discharged from the hospital. He was readmitted to the Nyaza Provincial Hospital on April 21, 1967, with recurrence of tumors in the left orbital cavity and right upper tibia and was treated with 100-mg doses of Cytoxan administered orally three times daily for 5 days (total dose, approx. 44 mg/kg).

On April 28, 1967, he was transferred to the Kenyatta National Hospital, Nairobi. In Nairobi it was found that the orbital tumor had destroyed the roof and lateral wall of that cavity and had extended into the left infratemporal fossa. The spleen was enlarged but an abdominal tumor was not evident. The upper half of the right tibia was radiologically uniformly tumorous. The histologic diagnosis was confirmed by left orbital biopsy on May 1, 1967. Tumor tissue was collected at the same time for test and culture and for an autochthonous irradiated tumor cell vaccine. Tumor tissue was subsequently collected for test and culture on May 8, 22, and 29, 1967. In view of the appearance of the first biopsy in the membrane fluorescence test described below, splenectomy was performed on May 8, 1967. The vaccine was given on May 17, May 24, and June 7, 1967. No tumor regression occurred after the second course of Cytoxan, which had been given at Kisumu, or following splenectomy and the first two vaccine inoculations.

On May 24, 1967, the left orbital tumor was thought to be increasing in size, and 200 mg cytosine arabinoside (NSC 63878) (1-β-D-arabinofuranosylcytosine hydrochloride) dissolved in 500 ml sterile distilled water (150 mg/sq m) was injected i. v. every 12 hours on May 25, 26, and 27, 1967. Marked regression of both tumors was evident by June 2, 1967, and this continued until June 11, 1967, when the child suddenly and un-

expectedly died. Permission for a postmortem examination was refused.

Direct Membrane Fluorescence Tests

Four biopsies were examined, obtained within a total time span of 28 days. As previously done (5, 7–9), all biopsies were immersed in Eagle's medium and packed in wet ice immediately after operation; they were shipped to Stockholm by the most direct air route. Tests were usually performed within 24 hours after biopsy. Viable cell supensions were prepared and processed for the indirect membrane fluorescence test (5, 7–9). As a first step, the cells are brought into suspension by mincing and shaking in tissue culture medium. This procedure regularly brings a large number of free cells into suspension containing lymphoblast-like cells of uniform size. The next step in the direct membrane test is to expose the cells to the various fluorescein-conjugated antiglobulin reagents to establish suitable, "background-free" reagent dilutions prior to the indirect tests. It was this preliminary test that revealed the exceptional behavior of the Daudi cells.

RESULTS

When cell suspensions from the first three biopsy samples (received May 2, 9, and 23, 1967, respectively) were exposed to the various conjugated reagents, 100 percent or nearly 100 percent full-ring membrane staining was obtained with the goat anti-IgM and the rabbit anti-kappa-lambda light chain conjugates (Table 1). The cells reacted up to high reagent dilutions (>1:1200 with the anti-IgM and 1:320 with the anti-light chain reagent). Anti-IgG and anti-IgA sera stained approximately 10–30 percent of the first two biopsy cells in 1:15 dilutions; this staining was less complete than with the IgM or anti-light chain reagents, however, and consisted mainly of small fluorescent dots and spots rather than of complete rings or sectors. The third biopsy showed a more pronounced IgG reactivity of an essentially similar appearance but of greater intensity and on a higher proportion (74%) of the cells. The fourth biopsy of this patient, received after chemotherapy on May 30, 1967, behaved differently. In contrast to the previous samples, this specimen was rather necrotic; only about 50% of the suspended cells were alive, as judged by the trypan blue staining test. No ring reaction was seen with the anti-IgM reagent and there were a few dots or spots on a minority (10–30%) of the cells with a high reagent concentration (1:10). Since the membrane test requires that the suspensions contain viable cells in a high frequency, the significance of the staining observed is more questionable than in the other cases. Nevertheless, it appeared that the relatively few viable cells showed a clear IgG and IgA surface reactivity (*cf.* Table 1) when the reagents were used in 1:10 dilutions. This reactivity was again of the dot-spot rather than the ring type and disappeared on further reagent dilution.

All four biopsy specimens were established as cultured cell lines, growing as stationary suspensions *in vitro*. They were designated NK-10a, 10b, 10c, and 10d, respectively.[3] Unlike biopsies from other patients that were explanted in a similar way,[3] the cells from the first three biopsies showed an immediate population increase within a week while the last biopsy behaved like most other biopsies, taking about four weeks before any population increase was apparent. As shown in Table 2, the Daudi cell lines derived from the first three biopsies maintained the high surface IgM reactivity, even after several weeks in culture with 50% of the end points up to 640-fold reagent dilutions or higher, and full ring staining of all or nearly all cells. In contrast to the biopsy tests, there was no sign of any membrane staining with the IgG or IgA reagents. The light chain anti-kappa-lambda reagent gave also a high titered, full-ring reaction with nearly 100 percent of the cells. Later, mono-

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Table 1

Antiglobulin specificity	Reagent dilution	Percentage of membrane-stained cells[a] 2.5 Biopsy I	9.5 Biopsy II	23.5 Biopsy III	Reagent dilution	30.5 Biopsy IV
IgG[b]	1:15	24, 36, 4	10,15, 19, 9	74	1:10	81, 84
					1:20	32
IgM[c]	1:40	100, 100, 100	100, 100	97[d]	1:10	33, 12
IgA[c]	1:15	38, 24, 11	22, 20	24	1:10	50, 72
					1:20	38
					1:40	0
Light chain[e] (kappa + lambda)	1:40	97, 100, 100	96, 100	100[f]	1:10	60
					1:20	25

Direct membrane fluorescence tests with viable biopsy cells from Daudi Onyango (K.C.C. No. 750), exposed to fluorescein-conjugated anti-human globulin reagents.

[a] Repeat tests on different aliquots from the same biopsy.

[b] Gift from Dr. J. B. Robbins (University of Florida).

[c] Hyland Laboratories, Los Angeles, California.

[d] 50% reactivity end point: >1,200 reagent dilution.

[e] Gift from Dr. G. Goldstein (University of Virginia).

[f] 50% reactivity end point: 320 reagent dilution.

Table 2

Antiglobulin[b] specificity	Reagent dilution	Line designation[a] NK-10a, Biopsy received May 2, Weeks in tissue culture 6[b], Percentage of membrane-stained cells	NK-10b, Biopsy received May 9, Weeks in tissue culture 4, Percentage of membrane-stained cells	NK-10c, Biopsy received May 23, Weeks in tissue culture 12, Percentage of membrane-stained cells	NK-10d, Biopsy received May 30, Weeks in tissue culture 3–8, Percentage of membrane-stained cells
IgG	1:15	0	0	0	0–0
IgM	1:40	100[d]	96[e]	100[f]	0–0
IgA	1:15	0	0	17	4–0
Light chain (kappa + lambda)	1:20	100	100[g]	96	n.t.
Kappa	1:40			100[f]	11
Lambda	1:20			14	

Direct membrane fluorescence tests with Daudi tissue culture lines, exposed to fluorescein-conjugated anti-human globulin reagents. n.t., not tested.

[a] See Footnote 3 in text.

[b] The same reagents were used as in Table 1. Anti-kappa and anti-lambda were obtained from Hyland Laboratories, Los Angeles, California.

[c] Cells of this line were also tested after 3 weeks in culture, followed by 3 months in frozen storage in dry ice in the presence of dimethyl sulfoxide, thawing, and reestablishment in culture. Membrane staining was observed on the thawed line with the IgM and kappa reagents, with end points exceeding 640. IgA, IgG, and lambda reagents did not stain.

[d] 50% end point of reactivity (reagent dilution) >1:640.

[e] 50% end point of reactivity (reagent dilution) >1:480.

[f] 50% end point of reactivity (reagent dilution) >1:80.

[g] 50% end point of reactivity (reagent dilution) >1:120.

specific light chain reagents became available and were tested against the line derived from the third biopsy (NK-10c), showing that all detectable light chain reactivity is of the kappa type.

Cells from the NK-10a line, derived from the first biopsy, were frozen in dry ice with dimethyl sulfoxide after 3 weeks in culture and thawed after 3 months in frozen storage at −79°C. Ring staining with the IgM and kappa reagents was fully maintained in the reestablished line (*cf.* footnote to Table 2). The NK-10d line derived from the fourth biopsy carried no detectable IgM or other immunoglobulin specificity on the cell surface.

Other suspension cultures available in the laboratory were also tested in a similar way. Anti-IgM, IgG, IgA, and light chain reagents occasionally gave a dot or spot-like membrane staining of a few cells, particularly when used at high concentrations, but no ring staining. Only a small minority of the cells showed this reaction, and no majority reaction resembling the Daudi cultures was seen. The following lines were included in these tests: the lines of Burkitt origin designated NK 6 (from the patient Annah, K.C.C. No. 733), NK 8 (Esther, K.C.C. No. 674), NK 9 (Silfere, K.C.C. No. 732), NK 11 (Margret, K.C.C. No. 759), all established at this laboratory,[3] Epstein's (2) lines EB 3, EB 4, and EB 5, Pulvertaft's line, Jijoye (13) and the non-Burkitt lines Daniels (12) and SKL3 (1).

Cytotoxic Effect of Immunoglobulin Antisera against Daudi's Biopsy Cells and Tissue Culture Lines

Table 3 shows the cytotoxic tests which were performed with the various immunoglobulin reagents against Daudi's biopsy cells and derived tissue culture lines. Normal spleen cells of Daudi and a number of established cell lines of Burkitt origin are shown for comparison. It will be seen that the biopsy of May 9 and May 23 displayed a high cytotoxic sensitivity to the anti-IgM and anti-kappa reagents, in agreement with the fluorescence test. The anti-IgG reagent did not kill the May 9 biopsy cells, whereas the May 23 biopsy showed considerable anti-IgG sensitivity as well. This is again in line with the fluorescence test (Table 1). The anti-IgA reagent was not cytotoxic for the May 9 sample, whereas the May 23 biopsy was sensitive for this reagent as well. This is a discrepancy, since the fluorescein conjugated anti-IgA reagent was negative against the May 23 sample in the membrane test. On the other hand, the fourth biopsy taken a week later (May 30), although unsuitable for the cytotoxic test because of the many dead cells present, *did* show an appreciable IgA and IgG coating in fluorescence, and the IgA discrepancy between the two tests on the May 23 sample may be a quantitative matter. The change in the reactivity of the different biopsy cells as a function of biopsy date is summarized in Chart 1*a* for direct membrane fluorescence and in Chart 1*b* for the cytotoxicity test. It will appear that the biopsies of May 2 and May 9 show evidence of IgM reactivity, whereas IgG and IgA give negative or borderline values. IgG coating appears on the biopsy of May 23 in addition to the IgM coating which is retained unchanged. The chemotherapy introduced on May 24 induced a radical change in the population. As already mentioned, a large proportion of the cells became necrotic and the tests reflect the behavior of the survivors only. These now have considerable IgG and IgA but little or no IgM surface reactivity.

At first we believed that all surface immunoglobulin reactivity represents *in vivo* coating from the outside, i.e., the patient's

Table 3

Name of donor	Source of target cells	Percentage of trypan blue-stained cells without antiserum	Anti-human immunoglobulin reagent: Anti-IgM[a] 1:25	Anti-IgM[a] 1:25	Anti-IgM[a] 1:625	Anti-IgG[a] 1:25	Anti-IgG[a] 1:125	Anti-IgG[a] 1:625	Anti-IgA[a] 1:25	Anti-IgA[a] 1:625	Anti-light[b] chain (kappa + lambda) 1:15	Anti-kappa[a] chain 1:25	Anti-kappa[a] chain 1:625	Anti-lambda[a] chain 1:25
Daudi (K.C.C. No. 750)	Biopsy May 9	14	0.90[c]			0			0.13			0.76		0
Daudi (K.C.C. No. 750)	Biopsy May 23	10	1.00		1.00	0.66		0.51	0.51	0.62		0.98	0.32	
Daudi (K.C.C. No. 750)	Normal spleen cells (May 9)	30	0									0		
Daudi (K.C.C. No. 750)	Tissue culture line NK-10a, derived from May 2 biopsy, 8 weeks in culture	7	0.85	0.70	0.40	0	0		0	0	0.44			
Daudi (K.C.C. No. 750)	Tissue culture line NK-10b, derived from May 9 biopsy, 7 weeks in culture	35	0.75			0.23			0.10	0.10		0.82	0.32	0.18
Daudi (K.C.C. No. 750)	Tissue culture line NK-10c, derived from May 23 biopsy, 8 weeks in culture	49	0.72			0			0			0.72		0
Daudi (K.C.C. No. 750)	Tissue culture line NK-10c, 10 weeks in culture	20				0					0.88	0.80		0.20
Daudi (K.C.C. No. 750)	Tissue culture line NK-10d, derived from May 30 biopsy, 4 weeks in culture	29				0					0			
Jijoye	Established Burkitt line (13)	8	0			0			0			0		0
Esther (K.C.C. No. 674)	Established line (NK-8),[d] 14 weeks in culture	17	0			0			0		0			
Silfere (K.C.C. No. 732)	Established line (NK-9)[d] 15 weeks in culture	30				0			0					
Annah (K.C.C. No. 733)	Established line (NK-6)[d] 16 weeks in culture	33	0			0			0					
Margret (K.C.C. No. 759)	Established line (NK-11)[d] 30 weeks in culture	27	0.23	0	0.23	0.23	0				0			
Issac (K.C.C. No. 788)	Established line (NK-15)[d] 6 weeks in culture	14	0.20	0.13	0	0.18	0					0		0

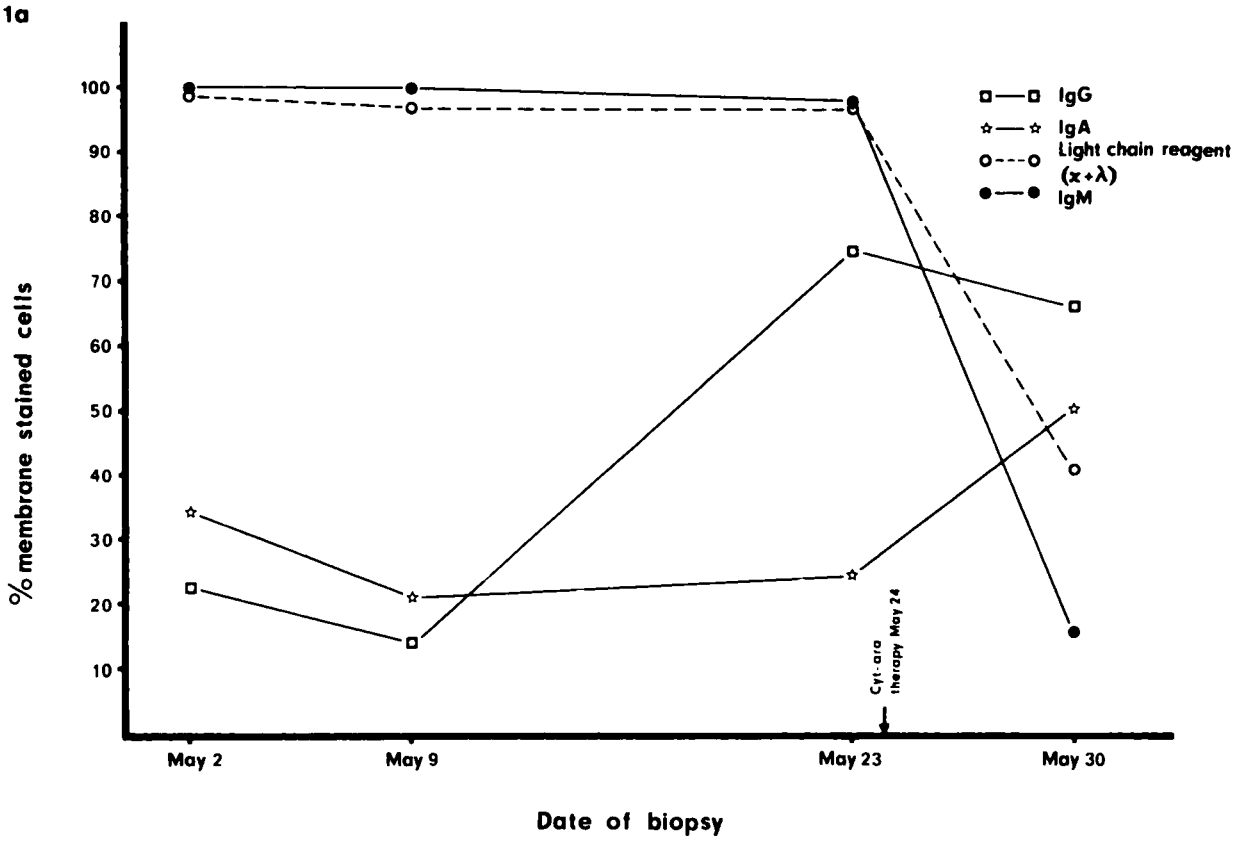
In vitro cytotoxic effect of rabbit anti-human immunoglobulin sera on Daudi's biopsy cells and tissue culture lines, in the presence of complement. A number of other established Burkitt lines are shown for comparison.

[a] Reagents obtained from the Department of Microbiology, University of Lund.

[b] Prepared and kindly provided by Dr. Gerald Goldstein, University of Virginia.

[c] The figures denote cytotoxic indexes, calculated by substracting the percentage of viable cells in the reagent-exposed preparation from the percentage viable cells in the sample exposed to complement alone, and dividing the difference with the latter. Fresh guinea pig serum was used as the complement source. Cell killing was assessed by trypan blue staining (2).

[d] See Footnote 3 in text.



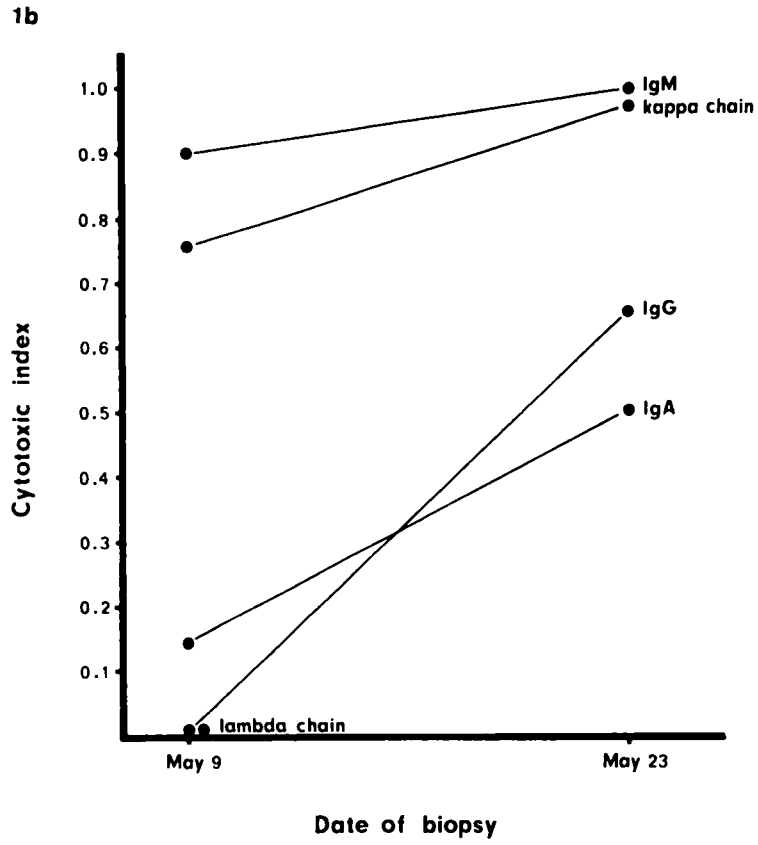


Chart 1. Immunoglobulin coating of Daudi's biopsy cells as a function of biopsy date. Chart 1*a* shows the direct membrane fluorescence tests. The reagent concentrations varied between 1:10 and 1:40. Each point is the mean of 2–8 separate tests. Chart 1*b* shows the results of the direct cytotoxicity test. All reagents have been used at a 1:25 dilution.

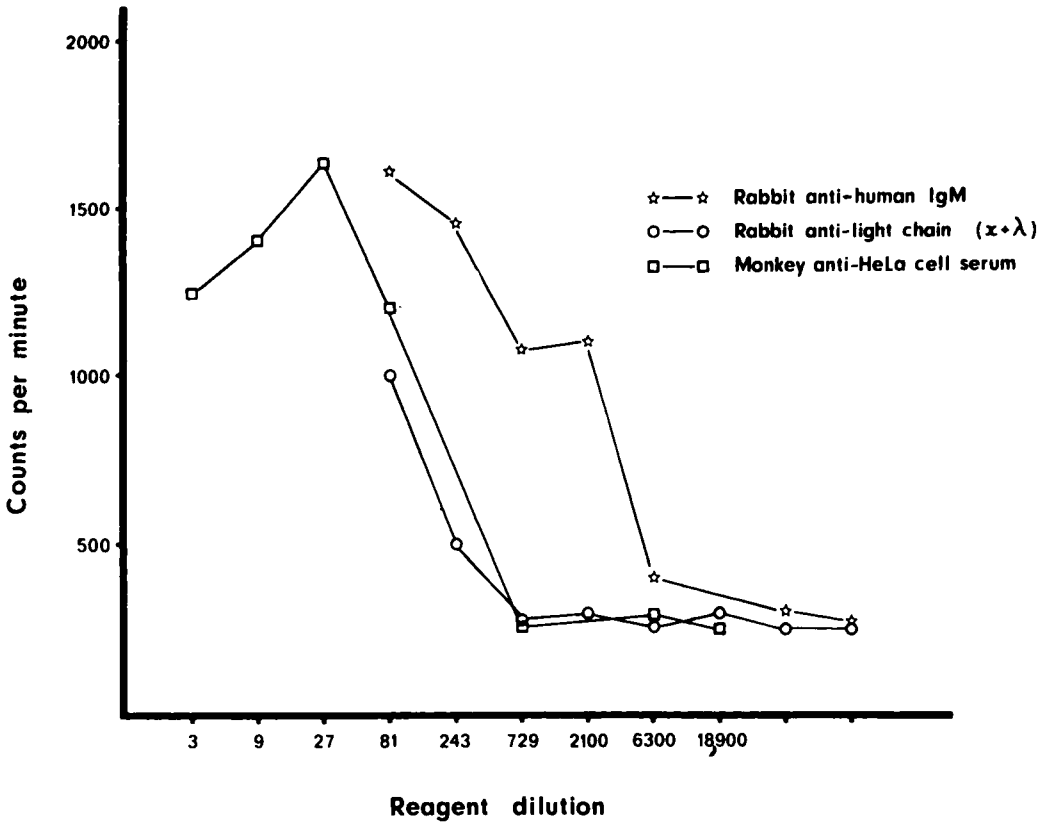


Chart 2. Cytotoxicity test, based on ^{51}Cr release (18), after *in vitro* exposure of the NK-10a cell line derived from the May 2 biopsy sample of Daudi, to anti-IgM and anti-light chain reagents, respectively, or to a heterologous rhesus anti-human (HeLa) cell serum, in the presence of complement. The cell line has been frozen at —79°C after 3 weeks in culture, kept in frozen storage for 3 months, and further *in vitro* passage during 3 additional months. In culture, the cells had a doubling time between 30 and 48 hours.

own serum. This became untenable, however, when we found that the IgM and the kappa light chain reactivity was maintained on the three cell lines established from the first three biopsies, whereas it was absent from the fourth line derived from the May 30 biopsy. As shown in Table 3, the cytotoxicity tests were entirely in line with the fluorescence tests on all four lines. It is particularly noteworthy that there was no evidence of any IgG or IgA coating preserved on the NK-10c or NK-10d lines derived from the May 23 and May 30 biopsies. The IgG and IgA reactivity of these biopsies is, therefore, probably best interpreted as being due to *in vivo* coating. Also of importance regarding the NK-10d line is the fact that it lacks appreciable IgM reactivity, in line with the fluorescence tests on the same line and on the biopsy material from which it was derived. On the basis of this reasoning, it was assumed, as the most reasonable explanation of the surface immunoglobulin patterns shown in Chart 1*a* and 1*b*, that IgM reactivity was essentially due to a production of material with both heavy and light (kappa) chain reactivity by the untreated Daudi cells themselves, with an accumulation of this material in the cell membrane, leading to a high degree of reactivity in the membrane fluorescence and the cytotoxicity tests as well. On the other hand, IgG and IgA, when present, were attributed to immunoglobulin coating from the outside.

The exceptional nature of the surface IgM specificity in the first three Daudi lines was further emphasized by the fact that sensitivity was maintained, both in the cytotoxicity and the membrane fluorescence tests, to high reagent dilutions. Chart 2 illustrates this for another type of cytotoxic test, based on the release of ^{51}Cr from labeled target cells, performed as described by Wigzell (18). It will be seen that the reactivity of the anti-IgM reagent exceeds even the efficiency of a heterologous monkey anti-human (HeLa cell) antiserum.

Table 3 also includes corresponding cytotoxicity tests on normal spleen cells from the patient Daudi and on five established culture lines of Burkitt origin, derived from other donors. No cytotoxicity was obtained against normal spleen cells with any of the reagents tested. At least one of the established Burkitt lines tested [Jijoye (3, 16, 17)] and probably others as well (R. van Furth, personal communication) secrete small amounts of immunoglobulins into the medium; nevertheless, with all the reagents tested they were completely negative in direct membrane fluorescence and cytotoxicity. So far, the first three Daudi lines represent a unique pattern of surface immunoglobulin reactivity among seven lines of Burkitt origin established by other workers (7) and a dozen lines newly established at this laboratory,[3] as judged by direct membrane fluorescence and/or cytotoxicity tests.

Table 4

Antiglobulin serum	Dilution	Percentage of killed cells after addition of serum fraction[a]	
		Albumin	19 S
Rabbit anti-IgM[b]	1:150	69	7
	1:750	44	9
	1:3000	33	9
Horse anti-IgM[c]	1:10	42	1
	1:50	52	3
	1:250	4	16
Goat anti-IgM[d, e]	1:10	50	3
	1:50	68	20
Goat anti-kappa[e]	1:10	54	12
	1:50	9	17
Goat anti-kappa[d, e]	1:10	80	30
	1:50	10	13

Inhibition of the cytotoxic effect of anti-human immunoglobulin sera against the Daudi cell line NK-10a by a 19 S fraction of normal human serum. 7×10^5 cells were mixed with 40 μl of antiserum in the dilutions indicated. Equal amounts of the serum fraction and of rabbit C′ were added. C′ was preabsorbed with peripheral white cells from chronic lymphatic leukemia. After 45 min incubation at 37°C, the test was read by the trypan blue method.

[a] Sephadex G-200 fractions of normal human serum (albumin or 19 S).

[b] Received from the Institute of Microbiology, University of Lund.

[c] Received from the Central Laboratory, Blood Transfusion Service, Amsterdam, Holland.

[d] Absorbed with cells from chronic lymphatic leukemia.

[e] Hyland Laboratories, Los Angeles, California.

Table 5

Serum fraction tested for inhibition	Antiserum reagent	
	Goat anti-IgM[a] (1:50)	Rhesus anti-HeLa (1:50)
19 S fraction, dilution		
1:4	18[b]	100[b]
1:8	16	100
1:16	45	100
1:32	62	100
7 S fraction, dilution		
1:4	81	100
1:8	80	100

Cytotoxic effect of a goat anti-human IgM serum and a rhesus anti-HeLa cell serum on Daudi cells of the NK-10a line after exposure to the 19 S or 7 S fraction of normal human serum. The test was performed in the same way as indicated in Table 4.

[a] Hyland Laboratories, Los Angeles, California.

[b] Percentage of killed cells.

Table 6

Dilution of the conjugated anti-IgM serum[a]	Percentage of membrane-stained cells after addition of serum fraction:	
	Albumin	19 S
1:20	87	6
1:40	85	4
1:120	83	2
1:400	83	0

Inhibition of the reactivity of fluorescein-conjugated goat anti-human IgM serum measured by the direct membrane fluorescence test. 40 μl of serum fraction were added to 5×10^6 cells. This was followed by 40 μl of the fluorescein-conjugated anti-IgM reagent. Subsequently, the test was processed as usual.

[a] Hyland Laboratories, Los Angeles, California.

Table 7

Cell source	Date of biopsy	Anti-IgM		Anti-IgG		Anti-IgA		Anti-light chain (kappa + lambda)		Anti-kappa chain,	Anti-lambda chain
		1:15, Fluorescence	1:25, Cytotoxicity	1:15, Fluorescence	1:25, Cytotoxicity	1:15, Fluorescence	1:25, Cytotoxicity	1:15, Fluorescence	1:25, Cytotoxicity	1:40, Cytotoxicity	1:10, Cytotoxicity
Biopsy	May 30	0	0	74	0.77	0	0.10	6		0.10	0.66
Biopsy	July 4	11	0	90	0	11	0	711			
Tissue culture after 3 weeks	May 30	0		0							
Tissue culture after 7 weeks	May 30	0	0.2	0	0.2			0	0		

Results of direct membrane fluorescence and cytotoxicity tests with two biopsy specimens and one derived tissue culture line, obtained from the Burkitt patient Margret (17 F, K.C.C. No. 759).

[a] Percent cells showing direct membrane fluorescence.

[b] Cytotoxic index, calculated as in Table 3.

Inhibition Tests

As a further test of the specificity of the reactions obtained with the IgM coated Daudi cell lines, inhibition tests were carried out with an albumin and a 19 S γ-globulin fraction isolated from normal human serum by fractionation on a Sephadex G-200. As shown in Table 4, the 19 S fraction completely inhibited the cytotoxic effect of three different anti-human IgM sera of rabbit, horse, and goat origin against the NK-10a line, derived from the first biopsy. The albumin fraction showed no inhibitory effect. A similar difference was found when a goat anti-kappa light chain reagent was used.

Conceivably, the 19 S fraction could have some anticomplementary activity, or its reaction with antibody might consume complement and thereby influence the cytotoxic reaction. As one specificity control, the 19 S and the 7 S fractions of the same normal human serum were tested for inhibitory activity against

Table 8

Donor						Anti-IgM		Anti-IgG		Anti-IgA		Anti-light chain (kappa + lambda)		Anti-kappa chain		Anti-lambda chain	
Name	Age (yr.)	Sex	K.C.C. No.	Cell source	Date biopsy received	1:15, Fluorescence	1:25, Cytotoxicity	1:15, Fluorescence	1:25, Cytotoxicity	1:15, Fluorescence	1:25, Cytotoxicity	1:15, Fluorescence	1:25, Cytotoxicity	1:10, Fluorescence	1:25, Cytotoxicity	1:10, Fluorescence	1:25, Cytotoxicity
Opasa	5	♂	766	Biopsy	June 27, 1967	11	0	0	0	14	0	4			0		0
				Biopsy	August 2, 1967	0		0		0		0		0		5	
				Biopsy	December 12, 1967	9	0	0	0			0		0		5	
				Tissue culture after 16 weeks	From biopsy of August 2, 1967	0		0				0					
Kibet	3	♂	767	Biopsy	June 27, 1967	24		4		27		8					
Issac	8	♂	788	Biopsy	August 15, 1967	3		7	0	5		0	0.09	4		3	
				Biopsy	August 29, 1967	5						21		8		3	
				Biopsy	September 12, 1967	0	0	0	0	3		0	0	34	0	5	0
				Tissue culture after 1 week	From biopsy of September 12, 1967			0									
				Tissue culture after 7 weeks		29	0.20	0	0.18					26	0		0
Katana	7	♂	801	Biopsy	September 26, 1967	32	0.11	23	0	3		17	0.10	30	0	8	0
Mwinzi	12	♂	786	Biopsy	September 12, 1967	100	0.66	7	0.64	26		93	0.63	100	0.43	5	0
				Biopsy	September 26, 1967	62	0.62	8		5		83	0.54	86	0.66	0	0.13
				Biopsy	October 17, 1967	74	0.87	22	0.73		0.69	77	0.73	92	0.79	0	0.66
Mkauni	6	♂	797	Biopsy	September 15, 1967	71	0.67	8	0.39	4		46	0.35	5	0.26	8	0.18
Abwao	6	♀	812	Biopsy	October 3, 1967	49	0.63	7	0.06	7	0.11	17	0.41	47	0.32	12	0.14
				Tissue culture after 1 week	From biopsy of October 3, 1967	4		0			0	0		6			
Ekesa	8	♂	816	Biopsy	November 14, 1967	82	0.88	24	0.30	23		6	0.35	61	0.35	20	0
				Tissue culture after 3 weeks	From biopsy of November 14, 1967	34		0				0					
				Tissue culture after 6 weeks		11		0						0			
Wilkister	7	♀	819	Biopsy	November 30, 1967	72	0.65	22	0	5	0	24	0.45	42		0	0.44
				Tissue culture after 4 weeks	From biopsy of November 30, 1967	15		0				0					

Direct membrane immunofluorescence and cytotoxicity tests on biopsies and some derived tissue culture lines from donors with histologically confirmed Burkitt lymphoma.

the goat anti-IgM serum and, in parallel, against the rhesus anti-HeLa cell serum as well. As shown in Table 5, the cytotoxicity of the anti-IgM serum, but not the anti-HeLa serum, was inhibited by the 19 S fraction. The 7 S fraction had no inhibitory effect. Even more significant was the fact that the direct membrane fluorescence obtained with the conjugated goat anti-human IgM reagent was also completely inhibited by the 19 S fraction (Table 6). The albumin fraction did not inhibit, not even at very high reagent dilutions (1:400). Since the membrane fluorescence test is not dependent on complement, the inhibitory effect of the 19 S fraction cannot be due to anticomplementary activity.

Fluorescence and Cytotoxicity Tests on Other Biopsies from Burkitt Lymphoma Patients and on Derived Tissue Culture Lines

Subsequent to the experience with the Daudi cell in May 1967, attempts were made to assess the frequency of the same or similar phenomena by testing biopsy preparations and, whenever possible, derived tissue culture lines from other Burkitt patients in a similar way. Two biopsies of particular interest were obtained from a 17-year-old Burkitt patient Margret (K.C.C. No. 759) on May 30, 1967, and on July 4, 1967. According to both fluorescence and cytotoxicity tests the first biopsy carried an IgG coating (Table 7). When a tissue culture line was established from this biopsy, and tested after 3 weeks in culture, no residual surface IgG or other immunoglobulin type could be demonstrated. This was taken to indicate that, in contrast to the first three biopsies from Daudi but in similarity to the fourth, the immunoglobulin present on Margret's biopsy cells was due to coating rather than production. The second biopsy of Margret also carried a strong IgG coating according to the fluorescence test, but the cytotoxicity test was negative this time. It may be speculated that the tumor cells acquired some blocking or anticomplementary factor in the interim. Subsequently, a number of other Burkitt lymphoma biopsies were tested in the same way. The results are shown in Table 8. It will appear that at least 2 patients (Opasa and Issac) lacked demonstrable immunoglobulin coating on repeated biopsies, taken in the course of several months, and on the derived tissue culture lines as well.

For the patients Kibet and Katana, the values are borderline, and there may be some slight immunoglobulin coating; but this is not certain. Mwinzi shows clear evidence of both IgM, IgG, and, on the last biopsy, possibly even some IgA coating. There is also considerable light chain reactivity. The difference between the kappa and lambda reactivities may be due to the relative strength of the reagents rather than to any real discrepancy. Unfortunately, no cell line could be established from this patient in culture, and the question of production *versus* coating remains unsettled. Mkauni, Abwao, Ekesa, and Wilkister all showed IgM coating, the latter with some IgG activity being also present. Tissue culture lines of Abwao, Ekesa, and Wilkister showed loss of the immunoglobulin coating. In the case of Abwao, this already occurred one week after culturing.

DISCUSSION

More than a dozen tissue culture lines carried in stationary suspension cultures have been derived from histologically confirmed Burkitt lymphomas in this laboratory.[3] Ten other lines, established by other workers, have also been imported and examined. Among all these lines, the behavior of the three Daudi lines, derived from the first three biopsies prior to chemotherapy, is unique in our experience so far in showing a 100% membrane staining with anti-IgM and anti-kappa light chain reagents, but not with anti-IgG, anti-IgA, and anti-lambda. Cytotoxic sensitivity *in vitro* to the IgM and kappa, and insensitivity to the other reagents, in the presence of complement, closely parallelled the fluorescence tests. Both reactions were demonstrable up to very high reagent dilutions. It seems to be of particular significance that the reactivity was also present on the corresponding biopsy preparations. In addition to the IgM reactivity, the third biopsy showed considerable IgG and probably some IgA reactivity as well, but, in contrast to the maintained IgM and kappa, the IgG and IgA disappeared rapidly in culture. The IgM-kappa reactivity was maintained during continuous culturing over more than 5 months, with cell doubling times varying between 30 and 48 hours, and has also withstood 3 months in frozen storage at −79°C followed by thawing and further culturing.

Our first interpretation of the strong surface IgM coating of Daudi's cells was influenced by the finding of antibodies in certain Burkitt patient sera, capable of reacting with Burkitt lymphoma cells (5, 7–10). We attributed it to antibody coating of antigenic cells, much like a positive Coombs reaction in certain autoimmune phenomena. Whereas this may be the correct explanation for the IgG and IgA coating of the third and fourth Daudi biopsy and the various immunoglobulin coatings of the other biopsies that disappeared rapidly in tissue culture as well, this is clearly untenable for the maintained surface IgM specificity of the first three Daudi lines. It must be concluded that these cells produce IgM with kappa chain specificity, and that this product accumulates in or on the cell membrane. The possibility that a small number of cells may secrete the immunoglobulin and that this adheres to the surface of the rest was considered but rejected when it was found that 1:1 artificial mixtures of the coated and noncoated (fourth) Daudi line resulted in mixed cell populations, with both components maintained in approximately equal proportions after growing into a large population.

Although the Daudi cells must be producing whole immunoglobulin molecules or parts of them with both heavy and light chain specificities, they seem to be different from ordinary immunoglobulin-secreting cells. The direct membrane fluorescence reaction characteristic of the Daudi cells, and the cytotoxic sensitivity to immunoglobulin reagents in the presence of complement, could not be demonstrated with any of the other Burkitt lines tested, although some of them have been shown (3, 16, 17) to secrete small amounts of immunoglobulin into the medium. Furthermore, preliminary findings by Dr. R. van Furth indicate that the Daudi lines do not secrete IgM into the medium (personal communication). One of us (G. Klein, unpublished observation) has compared intracytoplasmic

versus surface localization of immunoglobulins in myeloma cells and in Daudi cells, respectively. Intracellular localization was studied by direct immunofluorescence staining of air-dried, acetone-fixed smear preparations. Surface localization was assessed by parallel membrane fluorescence tests on living cell samples from the same cell suspension. An interesting contrast emerged: the myeloma cells showed massive intracytoplasmic but no membrane fluorescence with reagents directed against their heavy or light chain products. In contrast, the membrane-reactive Daudi cells showed little or no intracytoplasmic reactivity with the anti-IgM and anti-kappa reagents. In the dried, fixed state, their membrane reactivity was still demonstrable, but it now appeared in the form of widely scattered, broken membrane fragments spread over the flattened cells like scattered clouds in the sky. The diffuse, homogeneous cytoplasmic fluorescence which is characteristic of the myeloma cells and of the immunoglobulin-producing Burkitt cell lines (4) and which leaves only the area of the nucleus empty, was not seen in the Daudi cells.

Immunoglobulin-producing myeloma cells were also tested for cytotoxicity by exposing them to immunoglobulin reagents directed against their products in the presence of complement. No cytotoxic reactions could be registered.

Various methods involving, in one way or another, the demonstration of immunoglobulin specificities on cell surfaces have given positive results with normal lymphocyte populations. An interesting example is the demonstration of blast transformation after exposure to sera directed against allotype specificities (15) or various other immunoglobulin components, including heavy chains, light chains, and digestion fragments (14). This was interpreted to indicate the presence of Ig-components on the lymphocyte surface in spite of the fact that no immunoglobulins could be visualized by fluorescent technics on fixed and dried cells, i.e., by conventional cytoplasmic fluorescence. Indirect evidence indicates, in addition, that specific antigen-combining sites may be expressed on lymphocyte surfaces (11).

It is possible that the high concentration of IgM molecules on the surface of the Daudi cells, without demonstrable cytoplasmic IgM accumulation, reflects a corresponding type of specialization among normal lymphoid cells. Since the neoplastic transformation can obviously affect lymphoid cells of various types, it may occasionally pick up a rare normal lymphoid cell with a high immunoglobulin concentration on the cell surface, if such normal cells exist. The frequency of the hypothetical cell may be low and easily overlooked. It might be speculated that there are at least two distinct expressions of immunoglobulin synthesis in lymphoid cells: either a cytoplasmic-secretory process like in the plasma and myeloma cells, or an accumulation of immunoglobulin on the cell surface in the absence of detectable concentrations of cytoplasmic immunoglobulins like in the present Daudi cells. Further studies on the Daudi cells might provide clues about how one could demonstrate the hypothetical normal prototype and define its biologic characteristics.

Whatever the origin of the IgM coated Daudi cells, it is of interest that they vanished after massive necrosis has been induced in the tumor by cytosine arabinoside chemotherapy. The fourth biopsy, taken subsequent to the therapy, contained relatively few living cells, but these were covered by IgG and IgA and not by IgM, demonstrable only at relatively high reagent concentrations. The IgG and IgA reaction was probably due to immunoglobulin coating since it was lost as the cell line was established in culture. It must be assumed either that the original tumor cell population contained two cell types, one of which was lost subsequent to chemotherapy, or that one of the two cell types represents a normal rather than a neoplastic cell.

The question of how unique the Daudi cell type is among Burkitt cells in general cannot be answered at present. As shown above, cells carrying an IgM coating have also been found in other biopsy preparations, but this was not maintained in culture in the cases tested. Unless maintenance can be shown *in vitro*, it is not possible to conclude that coating is a result of production inside the cell rather than covering from the outside. Whereas prolonged *in vitro* maintenance of coating is evidence of production, the opposite is not necessarily true, however, since loss of a given specificity in culture may be due to the selection of a nonproducing cell line. Taken together with the demonstration of membrane-reactive antibodies in the sera of Burkitt patients (5, 7–10), our findings are probably best interpreted by assuming that both phenomena, production with surface accumulation and serum antibody coating *in vivo*, may occur. The secretion of small amounts of immunoglobulin into the medium, demonstrated in various Burkitt lines (3, 4, 16, 17), may represent still another pattern. The relative contributions of each of these processes remain to be disentangled in each case.

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