Proc. Nat. Acad. Sci. USA
Vol. 72, No. 4, pp. 1622–1626, April 1975

Cytotoxic Effector Cells Specific for B Cell Lines Transformed by Epstein–Barr Virus Are Present in Patients with Infectious Mononucleosis

(specific cell-mediated cytotoxicity)

ERIK SVEDMYR AND MIKAEL JONDAL

Department of Tumor Biology, Karolinska Institutet, S-104 01 Stockholm 60, Sweden

Communicated by George Klein, January 27, 1975

ABSTRACT **Peripheral lymphoid cells, from 12 cases of acute infectious mononucleosis (IM), were tested in a micro chromium-51 release assay for cytotoxic activity against a variety of cell lines that did or did not carry the Epstein-Barr virus (EBV) genome. Unfractionated lymphocytes from these patients were cytotoxic to both types of cell lines, as were lymphocytes from healthy individuals. If, however, lymphocytes bearing complement receptors were removed, the residual IM lymphocyte fraction was specifically cytotoxic for EBV-genome-carrying cell lines. The residual lymphocyte fraction in normal donors had no such effect. Heterophile-positive IM is caused by EBV, and these results indicate that, during the acute phase of this disease, patients harbor killer cells, probably T cells, which specifically kill EBV-genome-carrying B cells *in vitro*. No such specificity for EBV-genome-positive target cells was found in normal lymphocytes stimulated *in vitro* with autologous EBV-genome-positive lymphoblastoid cells. Such stimulated cells were highly cytotoxic to both genome-positive and negative lines after removal of complement receptor-positive lymphocytes.**

The Epstein-Barr virus (EBV) has been regularly associated with three human diseases; infectious mononucleosis (IM) (1), Burkitt's lymphoma (BL) (2), and nasopharyngeal carcinoma (2). The etiologic role of this virus is clearly established in IM, whereas, it remains suggestive but not proven in BL and nasopharyngeal carcinoma. IM is characterized by an intensive lymphoproliferation which, although it sometimes resembles the acute phase of leukemia, always is self-limiting and benign in character. This self-limitation *in vivo* is in contrast to the capacity of the patient's lymphocytes to proliferate indefinitely *in vitro* as continuous cell lines with surface characteristics typical for bone-marrow derived (B) lymphocytes (2). Such cell lines carry the EBV genome, as shown by nucleic acid hybridization or the presence of the EBV-associated nuclear antigen (EBNA) (2, 3). It would be essential to understand what mechanisms protect the IM patients from continuous lymphoproliferation.

Recent investigations have shown that a high proportion of the blast-transformed cells found in the peripheral blood of IM patients are thymus-derived (T) lymphocytes (4–7). As T lymphocytes are known to be the major defense mechanism in many viral infections (8), we have studied the capacity of lymphocytes isolated from IM patients to kill a variety of cell lines that do or do not carry the EBV genome. Furthermore, we have compared the target cell specificity of the IM lymphocytes with the specificity of lymphocytes stimulated *in vitro* with autologous EBV-genome-carrying cell lines.

Abbreviations: IM, infectious mononucleosis; EBV, Epstein-Barr virus; T cells, thymus-dependent lymphocytes; B cells, bone-marrow derived lymphocytes; BL, Burkitt's lymphoma; C′, complement.

In our first experiments we were not able to demonstrate any specificity of IM lymphocytes for EBV-genome-carrying cells, due mainly to a high background cytotoxicity of normal lymphocytes against both EBV genome positive and negative lines. Jondal and Pross have since shown that the "nonspecific" cytotoxicity of normal lymphocytes against EBV-genome-positive and negative cell lines, and also against lines of nonlymphoid origin, can be largely abolished by removing cells with complement (C′) receptors from the lymphocyte population (9).

By removing these "nonspecifically" cytotoxic lymphocytes in the IM system it has now been possible to demonstrate killer cells specific for EBV-carrying target cells.

MATERIALS AND METHODS

IM Patients and Normal Lymphocyte Donors. Heparinized blood from 12 cases of IM was received from the Roslagstull and Danderyd hospitals in Stockholm. The clinical diagnosis was confirmed with a positive Monospot test (Johnson and Johnson) (10, 11) in all cases. Seven of the patients had a Paul Bunnell and Davidson titer over 1/300, the others had titers ranging from 1/10 to 1/40. All patients were hospitalized cases, and were tested within 14 days after admission. Two cases were studied twice with 1 week's interval.

Lymphocytes from 3 EBV-seropositive normal individuals (ES, MJ, and NG) were used as controls. ES was used as reference control in seven out of eight experiments.

Lymphocyte Purification. Lymphocytes were isolated on Ficoll–Isopaque gradients (12), washed three to four times to remove thrombocytes, and then treated with carbonyl iron and magnetism (13). Usually $^1/_4$ of the lymphocytes were kept without further purification and the rest treated to remove C′ receptor positive cells by rosette sedimentation, as earlier described (13). This process yielded approximately as many fractionated as unfractionated lymphocytes.

Target Cells. These are listed in Table 1. All lines except three were kept on RPMI 1640 + 10% heat-inactivated (56° 30 min) fetal calf serum; U-698M and U-715M were kept on RPMI + 10% heat-inactivated newborn calf serum, and YAC-L on F13 + 10% calf serum. Cultures were grown in stationary flasks and subcultured three times weekly.

Chromium-51 Release Cytotoxicity Test. The assay was carried out as described earlier (14) with some slight modifications. The medium used was RPMI 1640 supplemented with 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer + 20% heat-inactivated fetal calf serum. The assay was done in a total volume of 0.14 ml in V-shaped

TABLE 1. *Cell lines used as targets in cytotoxicity tests*

Designation	Origin*	Cell characteristic	Presence of EBV genome	Refs.
KAPLAN	IM	B cell markers	Yes	See 14
ODUOR	BL	B cell markers	Yes	See 14
P3HR-1	BL	B cell markers	Yes	See 14
MAKU	BL	B cell markers	Yes	See 14
RAJI	BL	B cell markers	Yes	See 14
BJAB	BL	B cell markers	No	15
GC-BJAB	Subline of BJAB†	B cell markers	Yes	†
ES-W-1	N(EBV)	B cell markers	Yes	See 14
PS-B-1	N(EBV)	B cell markers	Yes	‡
RG-B-1	N(EBV)	B cell markers	Yes	‡
ES-2	N	B cell markers	Yes	—
K562	Chronic myelocytic leukemia	Neither B nor T markers	No	16
1301	Acute leukemia	Neither B nor T markers	No	17
Molt-4	Acute lymphatic leukemia	T cell markers	No	18
U-698M	Lymphosarcoma	B cell markers	No	19
U-715M	Lymphosarcoma	B cell markers	No	19
SIMPSON	Myeloma		No	18
GOODWIN	Lung cancer		No	20
YAC-L	Mouse Moloney lymphoma	T cell markers	No	21

* IM = infectious mononucleosis, BL = Burkitt's lymphoma, N(EBV) = derived from peripheral lymphocytes of healthy EBV-seropositive donors after addition of EBV *in vitro*, N = derived from peripheral lymphocytes of a healthy EBV-seropositive donor.

† Derived from BJAB by addition of EBV *in vitro*. Received from Dr. G. B. Clements.

‡ Received from Dr. W. Leibold.

microtiter plates (Linbro, New Haven, Conn.), using 1×10^5 or 3×10^5 effector cells and 6×10^3 labeled target cells. The plates were centrifuged at $200 \times g$ for 4 min to speed up initial contact. After 4–8 hr of incubation at 37° in 5% CO_2 atmosphere, 0.1 ml of the supernatant of each culture was carefully harvested. Cultures were usually set up in triplicate. Cytotoxicity was expressed as % lysis according to the following formula: % Lysis = $100\ (T - S)/(\text{Max} - S)$. T = release in test, S = spontaneous release in medium alone, Max = maximal release, obtained by lysis in distilled water overnight (or in rare cases the effect obtained by the highest lymphocyte dose used if this figure was higher). Maximal release was between 60 and 90% of the total isotope uptake, which varied between 500 and 4000 cpm. The spontaneous release of the ordinary EBV-genome-positive lines and of K562 seldom exceeded 30% of the release in distilled water. Some of the EBV-genome-negative lines sometimes gave an unacceptably high spontaneous release. This was particularly true for BJAB and its EBV-genome-positive subline GC-BJAB. No spontaneous release above 50% of the maximum was accepted. The mean and range of spontaneous release is given for each cell line in Figs. 1 and 2. The standard error of triplicate determinations of % lysis seldom exceeded 5 units.

In Vitro Stimulation of Lymphocytes with Autologous Lymphoblastoid Cell Lines. This was performed as described previously (14).

RESULTS

Table 2 illustrates the cytotoxic effect of lymphocytes from 10 IM patients against the EBV-genome-positive KAPLAN line (derived from IM) as compared with the genome-negative line K562. A normal lymphocyte control was included in each of the seven experiments. The lymphocytes of all IM patients were clearly cytotoxic to KAPLAN, whether C′ receptor-positive lymphocytes were removed or not. In contrast, most toxicity of the normal lymphocytes disappeared after removal of C′ receptor-positive cells. The EBV-genome-negative K562 was highly sensitive to the normal and to most IM lymphocytes, but little or no cytotoxic activity remained after depletion of the C′ receptor-bearing lymphocytes. There was a clear dose–response relationship for the cytotoxicity of fractionated IM lymphocytes. The magnitude of this cytotoxic effect varied between patients.

Figs. 1 and 2 summarize the results with lymphocytes from all 12 IM patients against several EBV-genome-positive and negative lines, with the higher effector cell number (3×10^5). A complete set of data is given for target cells KAPLAN and K562, in Table 2. These two cell lines were used as reference EBV-genome-positive and negative target cells respectively, in all experiments except no. 4 involving IM patients 4 and 5.

Fig. 1 shows that EBV-genome-positive lines were sensitive to IM lymphocytes and that this cytotoxic effect did not disappear after removal of the "nonspecifically" cytotoxic cells. The GC-BJAB was the one exception, since it behaved like its genome-negative "mother" cell, BJAB. There was a clear difference in cytotoxic activity between the lymphocytes of different IM patients and possibly also some difference in the sensitivity of the different lines. ES-W-1 and ES-2 appeared to be fairly insensitive, but this might have been due to the fact that they were only tested against IM lymphocytes with a comparatively low cytotoxic activity. The average effect of control lymphocytes from normal donors is given for each target cell below the histogram. Most of this cytotoxic effect disappeared after fractionation. When unfractionated IM lymphocytes were cytotoxic against EBV-genome-nega-

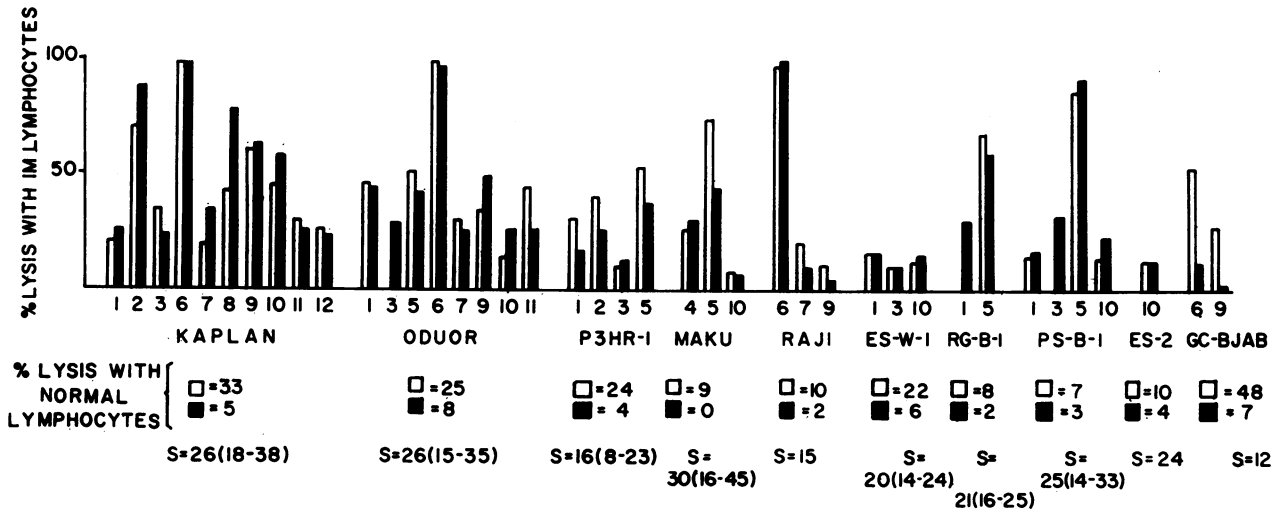

FIG. 1. Cytotoxicity of IM lymphocytes against EBV-genome-positive B cell lines. Results are expressed as % lysis (see Table 2) using 3×10^5 effector cells and 6×10^3 target cells. Open bars refer to results with unfractionated effector cells, filled bars to results with effector cells after removal of C′ receptor-positive cells. Numbers below bars indicate IM patient number. The average % lysis with normal lymphocytes before and after fractionation is given for each target cell below target cell designation. S = spontaneous release (given as % of maximal release) is given for each target cell as average and range (within parentheses). For origin and characteristics of target cells, see Table 1.

tive lines (Fig. 2) most or all toxicity disappeared after the removal of C′ receptor-bearing cells. The residual cytotoxicity occasionally left against some target cells was usually matched by similar results in the normal lymphocyte control as indicated by similar mean values for both.

Normal lymphocytes stimulated *in vitro* with autologous EBV-genome-positive lymphoblastoid cell lines showed extensive blast transformation, probably of T-cell origin (22). Such cells were approximately 10 times more efficient killers for the EBV-genome-positive and negative lines than were

TABLE 2. *Cytotoxic effect* of lymphocytes from 10 IM patients and a normal healthy individual against the EBV-genome-positive line KAPLAN and the EBV-genome-negative line K562*

Expt. no.	Donor of effector lymphocytes‡	KAPLAN†				K562†			
		Unfractionated§ lymphocytes		Fractionated¶ lymphocytes		Unfractionated§ lymphocytes		Fractionated¶ lymphocytes	
		1×10^5	3×10^5	1×10^5	3×10^5	1×10^5	3×10^5	1×10^5	3×10^5
I	IM 1	7	21	12	26	44	65	−4	−1
	ES	8	14	−2	6	93	100	—	−2
II	IM 2	41	70	56	88	44	80	0	1
	ES	57	89	—	10	95	100	—	30
III	IM 3	—	35	3	24	—	57	10	15
	ES	—	31	5	9	—	51	6	15
V	IM 6	91	99	90	98	84	100	7	11
	IM 7	11	19	16	34	77	95	4	2
	IM 8	28	43	45	78	60	90	5	5
	IM 9	33	61	35	63	88	93	7	9
	ES	—	16	—	3	—	100	—	6
VI	IM 6	36	69	35	73	68	83	6	6
	IM 9	23	32	17	31	74	84	10	13
	ES	12	25	—	6	86	97	9	11
VII	IM 10	29	45	37	58	19	35	3	−1
	ES	—	24	—	5	—	86	—	14
VIII	IM 11	—	30	—	24	—	97	—	5
	IM 12	—	26	—	24	—	76	—	5
	ES	30	45	6	0	99	99	2	−1

* Cytotoxic effect given as % lysis = $100(T - S)/(\text{Max} - S)$, where T = release in test, S = release in medium alone, and Max = maximal release (see *Materials and Methods*).

† Target cells were: KAPLAN (B cell marker derived from peripheral blood of IM patient), and K562 (without T or B lymphocyte markers). 6×10^3 target cells were used.

‡ IM-1, IM-2 . . . stands for 10 individual IM patients. ES = normal healthy EBV-seropositive donor.

§ Ficoll–Isopaque separated and carbonyl iron treated.

¶ C′-receptor positive cells removed by means of rosette sedimentation.

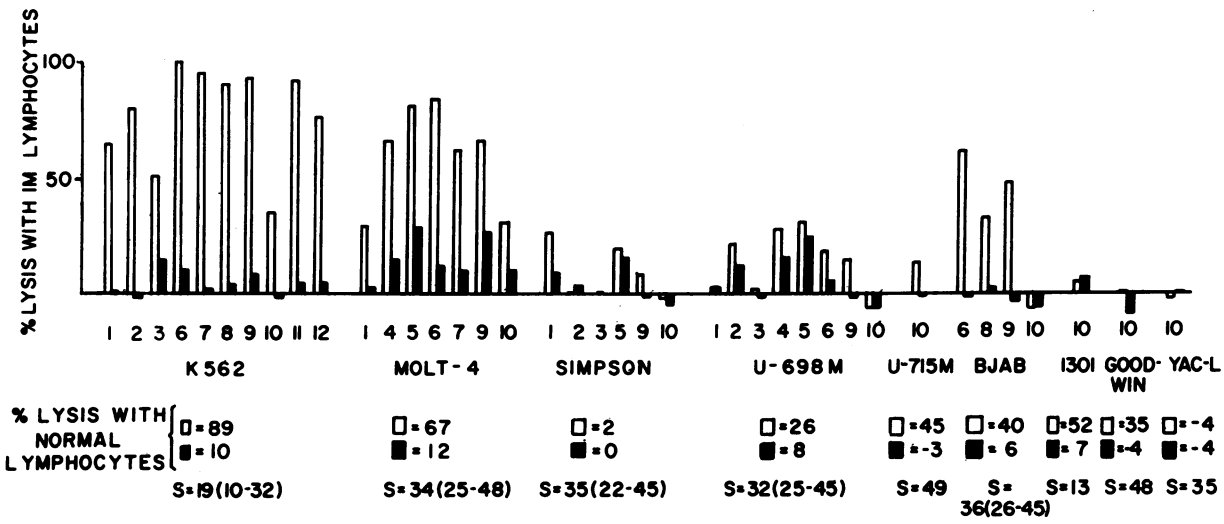

FIG. 2. Cytotoxicity of IM lymphocytes against EBV-genome-negative cell lines. Results are expressed as in Fig. 1. For origin and characteristics of target cells, see Table 1.

fresh IM lymphocytes. This cytotoxic activity did not disappear upon removal of C′ receptor-bearing cells, however, neither against EBV-genome-positive nor negative lines (Fig. 3).

DISCUSSION

Earlier reports (4–7) have demonstrated that a high proportion of the blast-transformed cells in the peripheral blood of IM patients are T-cell-derived. We and others have speculated that these T cells might be instrumental in bringing about the self-limitation of the disease (14, 7). Since EBV is known to be the cause of IM our approach to this problem was to investigate whether IM blood contained effector cells with cytotoxic specificity for EBV-genome-carrying target cells. We tested the capacity of IM lymphocytes to kill a panel of cell lines consisting of 10 EBV-genome-positive and 9 genome-negative lines. No specific cytotoxicity directed against the positive lines could be demonstrated if unfractionated lymphocytes were tested. However, cells with C′ receptors, which consist of B cells and an undefined lymphocyte subpopulation, have recently been shown to have the capacity to kill a variety of different cell lines in a nonspecific manner. It was, therefore, a logical step to remove these cells in order to investigate whether a specificity directed against EBV-genome-positive lines was hidden in the rest of the lymphocyte population. Thus, a specific killer cell population was found.

Although this study does not formally prove that the cytotoxicity directed against the EBV-genome-positive lines is a T-cell function, this conclusion is most probable. It is fair to assume that the cytotoxicity represents T-cell-mediated immunity directed against EBV-infected cells. It is interesting to note that this reactivity is directed against EBV-genome-carrying lines from both normal donors and from patients with BL or IM. This finding could be clinically important, as it has been shown that cell-mediated immunity can, under certain conditions, be transferred between individuals with so called transfer factor (23). If the structure on EBV-infected cell lines that is recognized by IM lymphocytes is also present on BL and nasopharyngeal carcinoma tumor cells, it would be reasonable to try treatment of such patients with transfer factor obtained from individuals with IM.

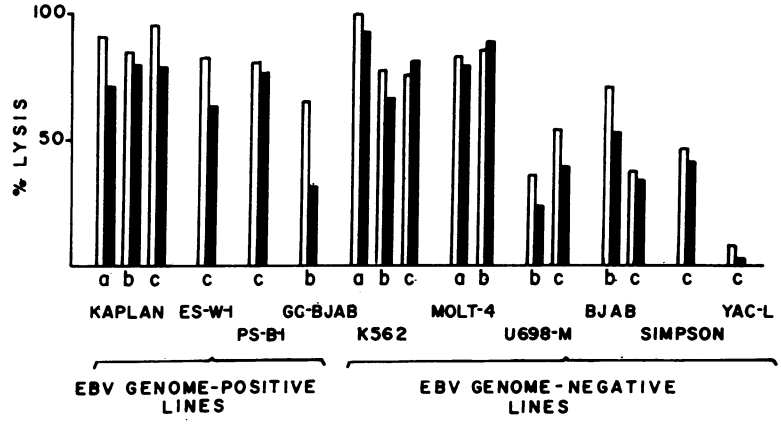

FIG. 3. Cytotoxicity of normal lymphocytes stimulated *in vitro* with autologous mitomycin-C-treated lymphoblastoid cell line, against EBV-genome-positive and negative target cell lines. Results are given as % lysis (see Table 2) using 3×10^4 effector cells and 6×10^3 target cells. Open bars refer to results with unfractionated effector cells, closed bars to results after removal of C′ receptor-bearing cells. Letters below bars indicate three separate experiments using the following lymphocyte-stimulator cell combinations for autologous stimulation: a = ES + ES-W-1, b = PS + PS-B-1, and c = ES + ES-W-1. These cultures were kept for 7 days *in vitro* and after this the effector cells produced were tested for cytotoxicity. Origin and characteristics of target cells are given in Table 1.

In earlier reports we have shown that lymphocytes stimulated *in vitro* with autologous EBV-genome-positive cell lines develop into killer T cells which indiscriminately kill EBV-genome-positive and negative lines (14, 22). We have now shown that this is so even if the lymphocyte population is treated to remove C′-bearing cells. This *in vitro* stimulation is thus not comparable to the *in vivo* stimulation occurring in an IM patient. Several explanations could account for this difference, such as lack of *in vivo* factors regulating self-recognition or appearance of new antigens on the cell lines *in vitro*. In any event, the *in vitro* stimulation system provides a specificity control for the IM system in showing that EBV-genome-containing target cells are not more sensitive to T-cell lysis *per se* than are EBV-genome-negative targets.

The present investigation provides evidence for the presence of effector cells in the peripheral blood of patients with acute IM, probably T cells, with specificity for EBV-genome-carrying B cells. Such effector cells may play an important role in the clinical manifestations as well as in the self-limitation of IM.

We thank Mrs. Linda van der Wal and Miss Ingrid Eriksson for technical assistance, Bill Sugden and George Klein for valuable criticism of the manuscript, and Drs. Per Olof Persson and Göran Sterner for supply of IM blood. This work was supported by Contract no. N01-CP-33316 within the Virus Cancer Program of the National Cancer Institute and Contract N01-CB-33859 within the Division of Cancer Biology and Diagnosis, National Cancer Institute, Department of Health, Education and Welfare. Grants were also received from Damon Runyon-Walter Winchell Cancer Fund (DRG-1064-C-II), the Swedish Cancer Society, and the Cancer Society of Stockholm.

1. Henle, W. & Henle, G. (1972) in *Oncogenesis and Herpes Viruses*, ed. Biggs, I. M., de-Thé, G. & Payne, L. N. (International Agency for Research on Cancer, No.2, Lyon), pp. 269–274.
2. Klein, G. (1973) in *The Herpes Viruses*, ed. Kaplan, A. (Academic Press, New York), pp. 521–555.
3. Reedman, B. M. & Klein, G. (1973) *Int. J. Cancer* **11**, 499–520.
4. Scheldon, P. J., Hernsted, E., Papamichail, M. & Holborow, E. J. (1973) *Lancet* **i**, 2253–2255.
5. Virolainen, M., Anderson, L. C., Lalla, M. & Von Essen, R. (1973) *Clin. Immunol. Immunopathol.* **2**, 114–120.
6. Yata, Y., Desgranges, C., de-Thé, G. & Tachibana, T. (1973) *Biomedicine* **19**, 479–483.
7. Denman, A. M. & Pelton, B. K. (1974) *Clin. Exp. Immunol.* **18**, 13–25.
8. Blanden, R. V. (1974) *Transplant. Rev.* **19**, 56–88.
9. Jondal, M. & Pross, H. (1975) *Int. J. Cancer*, in press.
10. Lee, C. L., Davidsohn, I. & Panczyszyn, O. (1968) *Amer. J. Clin. Pathol.* **49**, 12–18.
11. Wahren, B. (1969) *Amer. J. Clin. Pathol.* **52**, 303–308.
12. Böyum, A. (1968) *Scand. J. Clin. Lab. Invest.* **21**, Suppl. 97, 51–76.
13. Jondal, M. (1974) *Scand. J. Immunol.* **3**, 739–747.
14. Svedmyr, E., Deinhardt, F. & Klein, G. (1974) *Int. J. Cancer* **13**, 891–903.
15. Klein, G., Lindahl, T., Jondal, M., Leibold, W., Menézes, J., Nilsson, K. & Sundström, C. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 3283–3286.
16. Lozzio, C. B. & Lozzio, B. B. (1973) *J. Nat. Cancer Inst.* **50**, 535–538.
17. Foley, G. F., Lazarus, H., Farber, S., Uzman, B. G., Boone, B. A. & McCarty, M. C. (1965) *Cancer* **18**, 522–529.
18. Minowada, J., Ohnurna, T. & Moore, G. E. (1972) *J. Nat. Cancer Inst.* **49**, 891–895.
19. Nilsson, K. & Sundström, C. (1974) *Int. J. Cancer* **13**, 808–823.
20. Rabson, A. S., Rosen, S. W., Tashjian, A. H. & Weintraub, B. D. (1973) *J. Nat. Cancer Inst.* **50**, 669–674.
21. Cikes, M. & Klein, G. (1970) *Nature* **225**, 645–647.
22. Svedmyr, E., Wigzell, H. & Jondal, M. (1974) *Scand. J. Immunol.* **3**, 499–508.
23. Lawrence, H. S. (1969) *Advan. Immunol.* **11**, 195–266.