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Initiation of Oncogenic Transformation of Mouse Lymphocytes *In Vitro* by Abelson Leukemia Virus

(murine lymphoma/B-lymphocytes/plasmacytoma/karyologic markers/BALB/c mice)

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ABSTRACT **While C-type RNA viruses are known to induce leukemias and lymphomas, oncogenic transformation of lymphoid cells by them *in vitro* has not been reported. In this study, splenocytes from female BALB/c mice were infected *in vitro* with Abelson virus, a C-type RNA virus that induces nonthymic lymphomas and plasmacytomas in mice. The cells were transplanted into recipients of different karyotype, either male BALB/c mice or hybrid BALB/c × AL/N (CALF1) mice, which bear the *Rb(5.19)1Wh* translocation. Transplants of eight of the resulting tumors (one plasmacytoma and seven lymphomas) contained cells of donor BALB/c karyotype, indicating that transformation of splenocytes occurred *in vitro*.**

Although C-type RNA viruses have been associated with lymphocytic neoplasms in a wide variety of mammalian and avian species, little is known of the cellular mechanism by which they induce lymphomas. Even the question of whether these viruses transform their target cells directly or induce lymphomas by indirect means has not been settled. This is largely due to certain experimental drawbacks of the murine viruses generally used in such studies, for example, Gross passage A and Moloney leukemia viruses. First, there is generally a latent period of many months between time of infection and appearance of the tumor (generally a thymic lymphoma), a duration which obscures the oncogenic events (1). Second, there has been no system for transformation of lymphoid cells *in vitro*. An *in vitro* system would require a rapidly acting virus, the appropriate class of lymphocyte targets, a method for stimulating cellular DNA synthesis (required for viral replication and transformation) (2), conditions for keeping the lymphocytes alive in culture long enough to be transformed, and a means of determining whether the infected cells were oncogenically transformed. Transformation attempts *in vitro* with the thymic lymphoma viruses have not been successful, perhaps because present methodology does not permit maintaining thymocytes in culture long enough to be transformed by these relatively slow-acting viruses.

A different type of murine C-type lymphoma virus was isolated by Abelson and Rabstein from a Moloney leukemia virus-infected mouse that had been pretreated with prednisolone from birth (3). The Abelson virus (which is a mixture of Moloney leukemia virus and the Abelson component in a Moloney leukemia virus envelope) induces nonthymic lymphomas within as little as 3–4 weeks after injection of the virus into newborns of BALB/c and many other murine strains (3). Evidence has been obtained recently that the lymphomas induced by this virus may be of thymus-independent or B-lymphocyte origin (Sklar, Shevach, Green, and Potter, manuscript in preparation; Sklar, Premkumar, Singer, and Potter, manuscript in preparation). This virus also readily induces plasma cell tumors (plasma cells are end-stage B-cells) under certain conditions (4).

Since the target cells for transformation by Abelson virus appear to be thymus-independent lymphocytes in different stages of maturation, we attempted to transform these lymphocytes in cultures of splenic lymphocytes. These cultures contain 30–40% B-lymphocytes, which can be stimulated to DNA synthesis and division by several substances, including B-lymphocyte mitogens such as bacterial lipopolysaccharides (5).

To determine if oncogenic transformation had occurred, we transplanted the cultured spleen cells into mice to assay their malignancy. As we had found that these cultures replicate Abelson virus (unpublished data), it was necessary to distinguish between tumors that originate in donor cells from tumors arising *de novo* by infection of the recipient. This was accomplished by injecting the cells infected *in vitro* into recipient mice of different karyotype.

In these experiments, spleen cells treated *in vitro* with Abelson virus did in fact give tumors whose karyotypes show that they originated *in vitro*.

MATERIALS AND METHODS

Cells and Culture Methods. To maximize the possibility of obtaining plasma cell tumors, all mice used as spleen donors for tissue culture were injected with 0.5 ml of pristane (a light mineral oil) at 4–8 weeks of age (3). Spleens were harvested 20 or 30 days after pristane injection. Single cell suspensions were made and cultured by a modification of the method of Click *et al.* (6). The cultures were planted with 1×10^7 nucleated cells in 1 ml of medium, in 35-mm Falcon petri dishes.

The medium used (SK-10) contained RPMI-1640, 10% (v/v) fetal calf serum (Gibco) (heat-inactivated at 56° for 30 min), and 1% (v/v) of stock solutions of sodium pyruvate, nonessential amino acids (Microbiological Assoc.), and penicillin-streptomycin-glutamine (Flow Laboratories). 2-Mercaptoethanol was added to a final concentration of 50 μM. All cultures also contained *S. typhimurium* lipopolysaccharide W (Difco), 5 μg/ml. The cultures were maintained at 37° in a humidified 5% CO_2 atmosphere. They were fed every 2 days by addition of 0.15 ml of medium.

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Virus. The Abelson virus pool was a 10% (w/v) extract of primary tumors clarified by centrifugation and filtered through a 0.45-μm Millipore membrane filter. The pool had a tumorigenic $dose_{50}$ of $10^{3.6}$/ml in newborn BALB/c mice (Abelson component titer) and a Moloney virus titer of $10^{6.6}$ plaque-forming units/ml, as determined by the XC plaque test with BALB/c embryo fibroblasts (7). The virus pool also contains the LDH virus, an unrelated virus that is not required for lymphomagenesis by Abelson virus (unpublished data).

Transplantation System. Injection of mice with pristane before tumor cell inoculation greatly enhances transplantation of plasmacytomas (8) and Abelson lymphomas without affecting the rapidity or total incidence of lymphoma induction by Abelson virus (Sklar, Shevach, Green, and Potter, manuscript in preparation). Therefore, all recipient mice were injected intraperitoneally with 0.5 ml of pristane 1–6 weeks before injection of spleen lymphocyte cultures or tumor cells.

Karyotyping. Tumor-bearing mice were injected with colchicine, 1 μg/g of body weight. After 1.5 hr, cells were obtained from ascites and were karyotyped by standard methods, using Giemsa staining or quinacrine mustard banding (9, 10).

Experimental Design. A sample (0.05 ml) of the Abelson virus pool was added to each of the cultures to be infected, either at the time of planting (day 0) or after 24 or 48 hr.

At 4–7 days after planting (3–6 days after infection), floating and adherent cells (removed by scraping) were washed by centrifugation and injected intraperitoneally into recipient mice, either BALB/c or CALF1. Viable cells (3 to 6 $\times$ 10^6; by trypan blue exclusion) were injected into each mouse. In the experiments with CALF1 recipients, the control mice received a mixture of uninfected control cultures of cells derived from mice treated with pristane for both intervals (20 or 30 days) (total of 6 $\times$ 10^6 cells per mouse). To control for the possibility of tumors induced by graft-versus-host reactions (known to occur when parental splenocytes are injected into F_1 hybrids with a parent with a different H-2 marker), six pristane-treated CALF1 mice were each injected with 5 $\times$ 10^7 normal BALB/c splenocytes each week for 3 weeks.

Mice were examined every 2 days for the presence of ascites or adenopathy. Tumors that arose were classified by gross and microscopic pathology, and were tested for myeloma protein secretion into the ascites by agar electrophoresis. The heavy chain classes of the immunoglobulin produced by the plasmacytoma were typed by double-diffusion in agar (Ouchterlony).

In the initial experiments, the origin of the tumors was determined by infecting female (XX) spleen cells *in vitro*, injecting these cells into male (XY) mice, and scoring karyotypes of the resulting tumors for the presence or absence of the Y chromosome. Although male cells into female hosts would have been technically easier to karyotype, we hoped to avoid possible difficulties due to transplantation antigens on the male cells.

However, using the X and Y chromosomes as markers proved laborious, since formal karyotypes had to be prepared (as in Fig. 1). It was also difficult to obtain preparations uniformly good enough to screen enough metaphases for a reliable estimate of the percentages of host and donor derived cells.

Thus, we decided to use another chromosomal marker, the ***Rb(5.19)1Wh*** translocation (11). This is a Robertsonian translocation between chromosomes 5 and 19 found in all AL/N mice (12). These mice have 38 chromosomes, including the two large 5.19 translocations. A metaphase from a hybrid of AL/N with BALB/c (CALF1) is shown in Fig. 1. The translocation (*arrow*) is sufficiently evident to permit classifying metaphases unambiguously by direct microscopic observation. Thus, large numbers of metaphases can be easily screened, something that cannot be done reliably with the XY system.

We therefore infected BALB/c spleens *in vitro* and transplanted them to CALF1 mice. This direction of transplantation was chosen for three reasons. (*i*) The preliminary male–female studies indicated that BALB/c cells were susceptible to transformation *in vitro*. (*ii*) BALB/c is one of the few good strains for obtaining plasma cell tumors by infection *in vivo;* production of these tumors by transformation *in vitro* is one of our main goals. (*iii*) Any mouse strain would be potentially usable in such a system, since it could be transplanted into a hybrid of itself with AL/N.

All tumors that arose in the CALF1 mice were transplanted, either to CALF1 mice or to BALB/c $\times$ C57BL/6 (CBF1) mice. CBF1 mice ($H\text{-}2^d \times H\text{-}2^b$) were used as recipients for two reasons. (*i*) These mice would impose strong immunoselection pressure against any tumor or normal cells originating in the CALF1 ($H\text{-}2^d \times H\text{-}2^a$) mice, because of H-2 incompatibilities; only cells deriving from the BALB/c tissue cultures should transplant. (*ii*) CBF1 mice are relatively resistant to lymphomagenesis by Abelson virus (unpublished data: only three of 32 adult, pristane-primed CBF1 mice developed lymphomas from Abelson virus injection, none sooner than 47 days); this would minimize lymphomagenesis by Abelson virus released by the transplanted cells.

RESULTS

Initial Transplantation of Cells Infected In Vitro. Table 1 shows that in both groups of experiments, the mice receiving uninfected control cells did not develop tumors during the period of observation (groups A1 and B1). This was also true for the mice used to control for the possibility of tumors due to the graft-versus-host reaction (group B2).

In the groups injected with cells infected *in vitro*, tumors developed in four of seven mice in group A2, and 10 of 12 mice in groups B3–B6. The latent periods from cell injection until tumor diagnosis are similar to those obtained by injection of virus alone into adult, pristane-primed BALB/c mice. Thirteen of the 14 tumors were lymphomas by gross and microscopic examination. They arose as ascites tumors, with peritoneal seeding and marked lymphadenopathy in the peritoneum and various other sites. The gross pathological picture is similar to that previously reported in adult BALB/c mice infected with Abelson virus.

The single plasmacytoma that developed (A2-1) was also an ascites tumor, but with only peritoneal and retroperitoneal adenopathy. It was diagnosed both histologically and by agar electrophoresis of the ascites. Double-diffusion studies indicated that two different immunoglobulin classes were produced, IgA and $Ig\gamma_{2a}$.

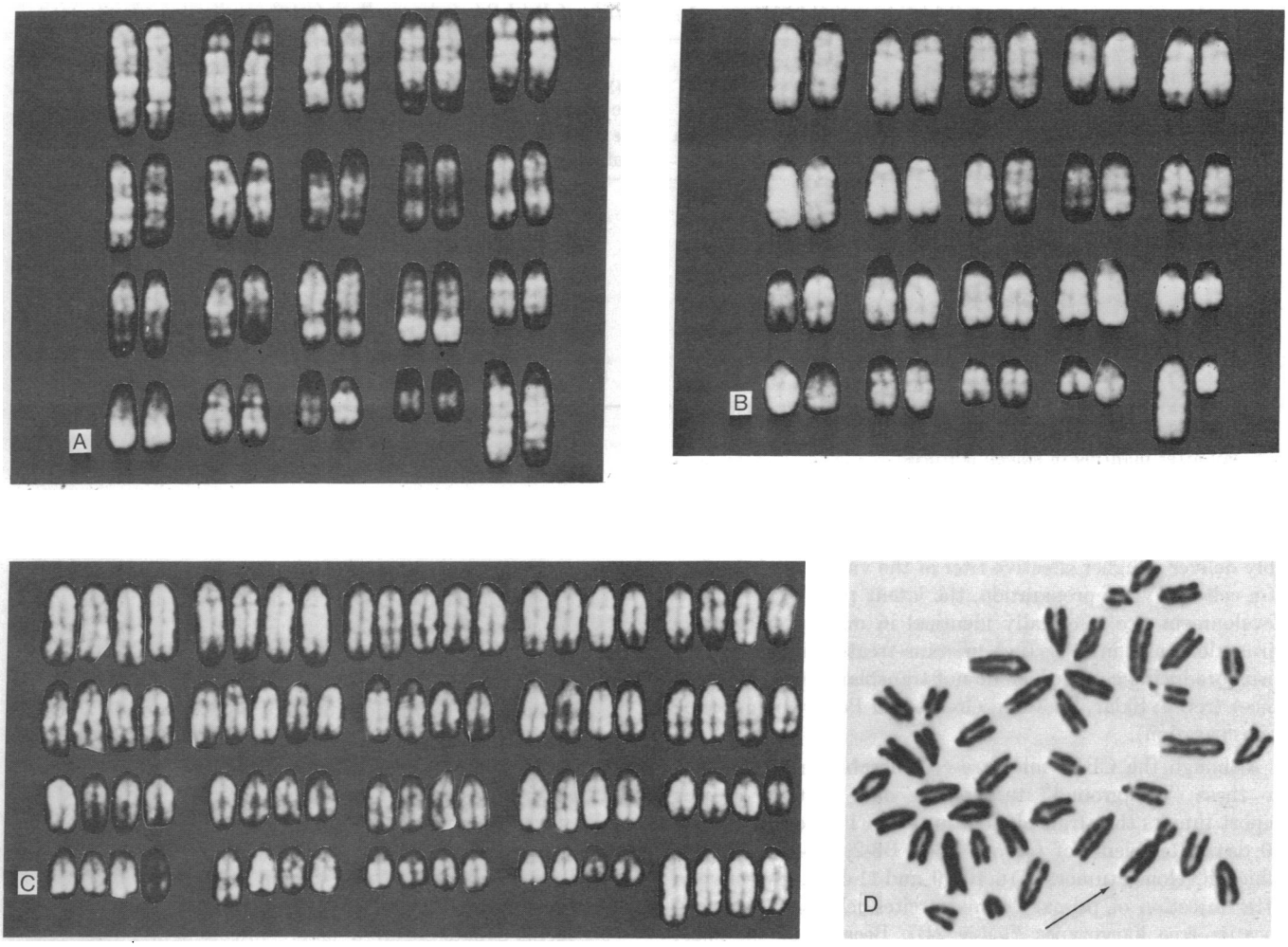

FIG. 1. (A–C) Quinacrine mustard banded karyotypes of plasmacytoma A2-1. (A) Female karyotype, 40 chromosomes; (B) male karyotype, 40 chromosomes; (C) female karyotype, 82 chromosomes. (D) Giemsa stain of metaphase from BALB/c × AL/N hybrid, 39 chromosomes, translocation (arrow).

Karyotyping: Female Cells into Male Mice (Group A). Attempts were made to karyotype ascites tumor cells from transplants of each of the four tumors in group A2. Adequate preparations were obtained only for the plasmacytoma, no. A2-1. A total of 25 metaphases were screened; the chromosome number ranges were 37–42 and 80–85. Nine quinacrine-stained cells were karyotyped in order to determine their sex. Table 2A shows that three were female (see Fig. 1A and C) and five were male (Fig. 1B). The sex of one cell could not be determined due to missing chromosomes.

It is not known whether the male cells in the tumors represent contamination with normal host cells or new tumor induction in the host by the virus produced by the transplanted cells. Whatever the source of the male metaphases, the female cells can only have originated *in vitro*.

Translocation Marker. In groups B3–B5, the tumors arising in the initial CALF1 recipients of infected spleen cells were transplanted again in CALF1 mice. Screening of metaphases from the ascites tumors that developed (Table 2B) showed that all of the cells in tumors B3-1 and B5-1 were without a translocation and had 40 chromosomes (normal BALB/c karyotype). Ascites tumors arising in two separate recipients of tumor B4-1 were analyzed. B4-1a yielded 98/100 cells with 40 chromosomes and without the translocation, while B4-1b yielded 50/50 cells with 39 chromosomes and with the translocation (normal CALF1 karyotype).

These results clearly show that tumors B3-1, B5-1, and B4-1a were of BALB/c origin; thus, they derive from cells infected *in vitro*. The other 2/100 cells of recipient karyotype in B4-1a might represent either host cell contamination or tumor induced *de novo* by the virus produced by the injected tumor cells. The latter is probably the case with the 50/50 CALF1 karyotypes in B4-1b.

Since we had found CALF1 mice to be quite sensitive to the Abelson virus (unpublished), we feared the possibility of a high background of virus-induced host tumor cells in the above experimental group. In an attempt to circumvent this possibility, we also transplanted some tumors (B6-1 to B6-4) from CALF1 to CBF1 mice. As previously mentioned, this strain should act as a filter, selectively permitting transplantation of cells descended from those infected *in vitro* while rejecting those from the CALF1 host (because of H-2 incompatabilities). Because CBF1 is also relatively virus resistant, the problem of new tumor induction by cell-borne virus should be minimized.

We have never observed an Abelson virus-induced tumor in less than 20 days in mice of any age or strain, and 26 days is the shortest latent period observed for an adult mouse

TABLE 1. *Tumor induction in BALB/c and CALF1 mice by injection of BALB/c spleen cells infected in vitro by Abelson virus*

Recipient strain of *in vitro* infected spleen cells	Group	No. of recipient mice	No. of days donor spleen pretreated with pristane	Abelson virus	Day* of infection of spleen culture	Day* of injection of cells	Day of diagnosis of tumor		No. alive and well at no. of days
							Lymphoma	Plasma cell tumor	
BALB/c	A1	4	30	0		5			4 @ 183
	A2	7	30	+	2	5	55,63,92	55	3 @ 187
CALF1	B1	4	20,30	0		4			4 @ 200
	B2†	6		0		†			6 @ 200
	B3	2	20	+	0	6	50		1 @ 200
	B4	4	20	+	1	5	32,35,37,42		
	B5	2	20	+	1	5	58		1 @ 200
	B6	4	30	+	0	6	34,44,51,56		

Blank spaces, not pertinent.
* Day after planting of spleen cultures.
† 5×10^7 BALB/c spleen cells were injected weekly for 3 weeks.

developing lymphoma. While cells producing virus can probably deliver a higher effective titer of the virus than injection of a cell-free virus preparation, the latent periods for tumor development are essentially identical in mice injected with virus alone and in mice (not pristane-treated) injected with virus-producing cells (which do not transplant readily in such mice) (ref. 4; Sklar, Shevach, Green, and Potter, manuscript in preparation).

Although the CBF1 mice are considerably more resistant to these "background" tumors, we only included in this report tumors that transplanted into CBF1 mice in less than 20 days. Recipients of tumors B6-1, B6-2, B6-3, and B6-4, which developed tumors at 16, 10, 19, and 11 days, respectively, after injection of primary tumor ascites cells, showed translocation-free karyotypes (Table 2B). Because of the short latent period, these tumors must represent growth of transplanted cells, and consequently must have originated *in vitro*.

DISCUSSION

These results indicate that we have obtained lymphomas from cells infected *in vitro* with a murine lymphoma virus. In the case of the plasmacytoma, two different immunoglobulins were produced and both male and female karyotypes were present. Without cloning, it is impossible to ascertain which immunoglobulin was synthesized by which cell. However, the presence of cells both with and without Y chromosomes suggests that the plasma cell tumor has a mixed origin, i.e., that some cells originated from *in vitro* transformation and others from *de novo* tumorigenesis in the host.

While it is clear that the tumors contained cells from the cultures infected *in vitro*, we do not yet know that the oncogenic events occurred before injection into mice. Since transformed lymphocytes are not necessarily distinguishable histologically from normal or mitogen-stimulated lymphocytes,

TABLE 2. *Karyotype data*

(*A*) *X/Y differences*

Mouse	Tumor type	Myeloma proteins	Transplant generation karyotyped	No. of metaphases karyotyped	No. of karyotypes that are		
					Female	Male	Indeterminate
A2-1	Plasma cell	A, γ_{2a}	2	9	3*	5†	1‡

(*B*) *Translocation*

Mouse	Recipient strain§	Day of diagnosis of transplantation in recipient mice	Karyotype of tumor diagnosed on day no.	No. of metaphases examined	No. of metaphases with ***Rb(5;19)1*** translocation
B3-1	CALF1	25,25,41	25	36	0
B4-1	CALF1	22(a),41(b),51	22(a)	100	2
			41(b)	50	50
B5-1	CALF1	28,36,52	28	50	0
B6-1	CBF1	16,19,21,21	16	76	0
B6-2	CBF1	10,10,11	10	70	0
B6-3	CBF1	11,11,14	11	50	0
B6-4	CBF1	19,24,32	19	50	0

* Chromosome numbers of 39, 40, and 82.
† Four had 39–42 chromosomes, one had 85 chromosomes.
‡ Chromosome number of 37.
§ All were inoculated with tumor that arose in initial CALF1 recipient of splenocytes infected *in vitro*.

proof that transformation can take place entirely *in vitro* awaits development of long-term culture of these lymphocytes transformed *in vitro*. Optimal conditions for culture, infection, and long-term cell-line growth await development. While we have obtained growth of Abelson virus-treated lymphocytes *in vitro* for as long as 3 months, we have not yet transplanted them.

One of the principal potential values of this system is that it provides a means of studying the mechanism by which a C-type RNA lymphoma virus can oncogenically transform lymphocytes, the cell type with which C-type virus expression and tumorigenesis are most often associated. However, it should be noted that transformation of lymphocytes by the Abelson virus *in vitro* does not bear on the question of whether the thymic lymphoma viruses directly transform their target lymphocytes.

Since there is evidence that the Abelson virus transforms B-lymphocytes *in vivo*, the *in vitro* system described here should provide a means of studying viral transformation of a well-defined cell population with more available functional and genetic markers than any other cell type. Cell separation procedures are available to select homogeneous lymphoid subpopulations for transformation. For example, cells with receptors for a given antigen could be separated from lymphoid tissue from mice immunized to that antigen by using the cell separator of Herzenberg and coworkers (13, 14). DNA synthesis could then be stimulated by lipopolysaccharide or other mitogens, or by the specific antigen, followed by transformation with Abelson virus. In the absence of the cell separator, specific antigen could be used to stimulate the desired subpopulation of B-lymphocytes. Resulting tumors would be tested both for specific receptor and production of specific antibody. The effect of virus transformation on B lymphoid surface antigens, antibody production, B-cell maturation, and other B-cell functions could all be studied independently of host antiviral and antitumor mechanisms. Virus transformation of such antigen-specific cells may give rise to lymphomas and plasmacytomas with known antibody-binding activity.

We thank Dr. Michael Potter for analyzing the myeloma proteins and for advice and help during this work. We also thank Drs. Helen and Hayden Coon for preparing the quinacrine mustard-stained karyotypes.

Note Added in Proof. It has recently come to our attention that Lieberman and Kaplan [(1966) *Nat. Cancer Inst. Monograph* **22**, 549–554] presented evidence that initiation of thymocyte transformation may have occurred following exposure to radiation leukemia virus *in vitro*.

1. Gross, L. (1970) in *Oncogenic Viruses* (Pergamon Press, Oxford), pp. 328–352.
2. Temin, H. (1967) *J. Cell. Physiol.* **69**, 53–63.
3. Abelson, H. T. & Rabstein, L. S. (1970) *Cancer Res.* **30**, 2213–2219.
4. Potter, M., Sklar, M. D. & Rowe, W. P. (1973) *Science* **182**, 592–594.
5. Watson, J., Epstein, R., Nakoinz, I. & Ralph, P. (1973) *J. Immunol.* **110**, 43–62.
6. Click, R. E., Benck, L. & Alter, B. J. (1972) *Cell. Immunol.* **3**, 264–278.
7. Rowe, W. P., Pugh, W. E. & Hartley, J. W. (1970) *Virology* **42**, 1136–1139.
8. Potter, M., Pumphrey, J. & Walters, J. (1972) *J. Nat. Cancer Inst.* **49**, 305–308.
9. Miller, O. J., Miller, O. A., Kouri, R. E., Alderdice, P. W., Dev, V. G., Grewal, M. S. & Hutton, J. J. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1530–1533.
10. Caspersson, T., Zech, L. & Mooest, E. (1970) *Science* **170**, 762.
11. White, B. J., Tjio, J. H., Van de Water, L. C. & Crandall, C. (1972) *Cytogenetics* **11**, 363–378.
12. White, B. J. & Tjio, J. H. (1974) *Cytologia*, in press.
13. Hulett, H. P., Bonner, W. A., Sweet, R. G. & Herzenberg, L. A. (1973) *Clin. Chem.* **19**, 813–816.
14. Julius, M. H., Masuda, T. & Herzenberg, L. A. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1934–1938.