Membrane antigen on Epstein–Barr virus-infected human B cells recognized by a monoclonal antibody

(hybridoma/immunofluorescence/cytotoxic T cells)

S. F. SLOVIN*, D. M. FRISMAN†, C. D. TSOUKAS*, I. ROYSTON†, S. M. BAIRD†, S. B. WORMSLEY†, D. A. CARSON*, AND J. H. VAUGHAN*‡

*Department of Clinical Research, Scripps Clinic and Research Foundation, and †Departments of Pathology and Medicine, University of California at San Diego, La Jolla, California 92037

Communicated by Ernest Beutler, December 31, 1981

ABSTRACT This paper describes a monoclonal antibody (B532) that detects a membrane antigen present on $\geq 95\%$ of the B cells from lines carrying the Epstein–Barr virus (EBV) genome. Evidence suggesting that B532 is EBV-related was originally obtained by using a cell-binding radioassay with different cell line substrates. Immunofluorescence and cell-sorter analysis confirmed that the antigen was present in high density on all EBV-infected lymphoblastoid B-cell lines, but not on EBV-negative B-, T-, myeloid, or null cell lines. Isolated normal peripheral blood B and T lymphocytes and monocytes failed to bind B532. The monoclonal antibody did not inhibit *in vitro* EBV infection nor did it block the killing of EBV-infected targets by cytotoxic T lymphocytes. The cell surface antigen recognized by B532 was shown by immunoprecipitation to have a molecular weight of $\approx 45,000$.

A number of monoclonal antibodies to Epstein–Barr virus (EBV)-associated membrane antigens have been described (1–3). These are directed against glycoprotein antigens found both on the virion and on EBV-producer cell lines (1, 2). In this report, we describe a monoclonal antibody derived from the spleen of a mouse injected with the EBV-infected, nonproducer lymphoblastoid B-cell line RPMI 8392. This monoclonal antibody, B532, strongly reacts with its parent B-cell line, RPMI 8392, and with other EBV-infected B-cell lines but not with its corresponding parent T-cell line, RPMI 8402, nor with non-EBV-infected B-cell lines.

MATERIALS AND METHODS

Immunization Protocol for Hybridomas. Female BALB/c mice were hyperimmunized with the RPMI 8392 B-cell line (4) by four intraperitoneal injections of 1×10^7 cells at 10- to 18-day intervals, followed by two intravenous injections of 1×10^7 cells spaced 3 mo apart.

Production of Hybridomas. One mouse spleen was removed, dispersed into a single-cell suspension, and then fused with the murine myeloma cell line P3-NS1/1-Ag4-1 (5) by the method of Galfre *et al.* (6). Then the cells were suspended in hypoxanthine/aminopterin/thymidine medium and dispensed into 96-well plates (Costar no. 3524, Cambridge, MA) at a density of 1, 2, or 3×10^5 cells per well. Hybrid colonies appeared 10–14 days later. Supernatants were screened for binding to the immunizing line RPMI 8392 and to the autologous T-cell line RPMI 8402 (4) by an indirect binding assay. Of 203 hybridomas analyzed, 26 bound to RPMI 8392 but not to RPMI 8402. One of these hybridomas, B532, also failed to react with EBV-negative B-cell line LNPL and, therefore, was chosen for further study.

Cloning of Hybridoma B532. The monoclonal B532 was subcloned by limiting dilution in a 96-well plate in the presence of a normal murine spleen cell feeder layer. Ten subclones were retested and all showed the same binding characteristics as with the original clone. Three of these were grown up to 10^6 cells per ml in 200-ml portions. These cells were then frozen down, and the supernatants were used as the monoclonal antibody. The monoclonal antibody is an IgG1 proteoin. For routine use, it was digested with pepsin by the protocol of Nisonoff (7) to produce $F(ab')_2$ fragments.

Preparation of Cells for Assay: Normal Human Cells. Peripheral blood leukocytes (PBLs) from normal healthy donors were collected, separated as described (8), and then frozen in dimethyl sulfoxide and liquid nitrogen until use. In addition, EBV lines were made from several of the donors by infection of their PBLs with the B95-8 strain of EBV as described (8). All cell lines were maintained in RPMI 1640 with 10% fetal calf serum supplemented with 10 mM L-glutamine, 100 units of penicillin per ml (Flow Laboratories, McLean, VA) and 100 μ g of streptomycin per ml (Flow Laboratories).

Binding Assay of Culture Supernatants. A solid-phase indirect assay (9) was used to determine the reactivity of the B532 antibody against different cells. In brief, 96-well round-bottom plates (Linbro Division, Flow Laboratories) were precoated with 0.5% bovine serum albumin in isotonic phosphate-buffered saline (pH 7.4) by incubation at room temperature overnight. After removal of the albumin solution, 2×10^5 cells were added per well in triplicate with 25 μ l of antibody B532 or phosphate-buffered saline/bovine serum albumin and allowed to incubate at room temperature for 2 hr. After washing the cells four times with phosphate-buffered saline by spinning the plates in a Sorval GLC table-top centrifuge at 1500 rpm for 5 min, 10 μ l of 125 I-labeled rabbit anti-mouse IgG (20×10^4 cpm) was added to each well. After incubation for 2 hr at room temperature or overnight at 4°C, the cells were further washed and dried, and the individual wells were counted.

Immunofluorescence. Cells were stained in suspension with a $F(ab')_2$ preparation of B532, followed by a $F(ab')_2$ fluorescein isothiocyanate-conjugated goat anti-mouse IgG. MOPC-21, an IgG1 (κ)-secreting myeloma (Bionetics, Kensington, MD), also was digested with pepsin and used as a negative control at the same protein concentration as was B532 (1 mg/ml). In addition, PBLs and cell lines were stained for the presence of Ia antigens by using monoclonal antibody L243 (10) and for HLA-framework antigens by using monoclonal antibody L368 (10) (both kindly provided by Robert Fox, Scripps Clinic). The presence

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: EBV, Epstein–Barr virus; PBL(s), peripheral blood leukocyte(s); HLA, human histocompatibility antigen; LYDMA, lymphocyte-defined membrane antigen.

‡To whom reprint requests should be addressed.

of EBV was shown by positive staining for the Epstein-Barr nuclear antigen by using the anti-complement immunofluorescence assay of Reedman and Klein (11).

The Cell Lines. Cell lines used were Wi-L2 (12), BJA-B (13), BJA-B-EBV (13), CCRF-CEM (14), NALL-1 (15), NALM-1 (16), HSB-2 (17), K562 (18), ML-1 (19), HPB-ALL (20), Molt-4 (21), HL-60 (22), Ramos (23), B85 (24), and NALM-6 (25). Loukes, an EBV-negative line from an American patient with Burkitt lymphoma, was obtained from E. Kieff (University of Chicago). LNPL, another EBV-negative B-cell line, was recently isolated from a patient with a diffuse large-cell lymphoma (26). The EBV-negative Ramos B cell line was kindly provided by I. Trowbridge (Salk Institute). EBV-infected B cell lines AV-EBV and AT-EBV were established in the University of California and Scripps Laboratories, respectively, by the infection of peripheral blood lymphocytes with the Epstein-Barr virus isolate B95-8.

Virus Neutralization Studies. B lymphocytes were prepared from PBL isolated by Hypaque/Ficoll centrifugation and passed over a rabbit anti-human Fab' immunoabsorbent column as described by Chess and Schlossman (27). Recovery was greater than 90%. The purified B cells were seeded into multiple triplicate wells of a Linbro 96-well microtiter plate at a density of 1×10^6 cells per 0.1 ml of RPMI 1640/20% fetal calf serum/L-glutamine/Penstrep. The cells were incubated sequentially with 0.1 ml of (i) a preincubated (overnight at 4°C) mixture of 0.5 ml each of monoclonal activity B532 and of a 1:5 dilution of purified B95-8 virus (2); (ii) monoclonal antibody B532 that was allowed to incubate with the cells for 1 hr at 37°C before 0.1 ml of a 1:10 dilution of B95-8 virus was added; (iii) 1:10 dilution of B95-8 virus, which was incubated with the cells

for 1 hr at 37°C and removed before 0.1 ml of monoclonal antibody B532 was added; (iv) monoclonal antibody B532 alone; and (v) a 1:10 dilution of B95-8 virus. All wells were brought to a final volume of 0.3 ml with the addition of medium. Cells were then allowed to go without further feeding for 6 days, after which time they were fed twice weekly by removal of 0.2 ml of medium and then adding fresh medium. Unneutralized virus induced the outgrowth of cells, with visible clumping after 14 days in culture.

Immunoprecipitation. Lactoperoxidase-catalyzed surface iodination and extraction of cell lines RPMI 8392, RPMI 8402, and Wi-L2 were carried out in 1% Triton X-100/6 M urea; the extracts were immunoprecipitated with B532 antibody concentrated 10-fold and with rabbit anti-mouse IgG (Miles) and were analyzed on NaDodSO₄/polyacrylamide gel electrophoresis as described by Baird (28). Membrane extracts were precleared with normal rabbit serum and sheep anti-rabbit IgG serum (28). Molecular weights of immune precipitates were determined by using six known standards that were provided by Bio-Rad and then stained with Coomassie blue. Those were: phosphorylase b (M_r , 92,500; relative mobility, 0.16), bovine serum albumin (M_r , 66,000; relative mobility, 0.27), ovalbumin (M_r , 45,000; relative mobility, 0.49), carbonic anhydrase (M_r , 31,000; relative mobility, 0.69), soybean trypsin inhibitor (M_r , 21,500; relative mobility, 0.93), and lysozyme (M_r , 14,400; relative mobility, 0.99).

Cytofluorographic Analysis of PBLs and EBV-Infected Lymphoblastoid B-Cell Lines. Normal human PBLs and EBV-infected lymphoblastoid B-cell lines were obtained as described. Cells (1×10^7) were stained with B532 and MOPC 21, followed by fluorescein-conjugated goat anti-mouse IgG F(ab')₂ fragments, and then were washed and resuspended in 1 ml of 1% formalin/phosphate-buffered saline. Cells were filtered and then analyzed with an Ortho Cytofluorograf 50H (Ortho In-

Table 1. Plate binding assay: Screening of supernatant antibody B532 on cell panel

Cell lines	Binding, * cpm
Cultured cells	
B cells	
RPMI 8392	2,458 ± 85
B85	2,159 ± 150
Wi-L2	1,585 ± 113
BJA-B-EBV	919 ± 49
BJA-B†	479 ± 10
LNPL†	235 ± 30
T cells	
RPMI 8402	198 ± 3
Molt-4	ND
CCRF-CEM	378 ± 46
NALM-6	318 ± 27
HSB-2	209 ± 42
HPB-ALL	356 ± 10
Myeloid cells	
HL-60	ND
K562	316 ± 46
ML-1	300 ± 19
Medium background†	
Blank well	120 ± 6
On cell lines	112 ± 8
Normal PBLs and EBV-infected cells	
AV-PBL	377 ± 91
AV-EBV	880 ± 51
AT-PBL	398 ± 5
AT-EBV	2,815 ± 10

* Mean ± SD.

† EBV-negative lines.

‡ Bovine serum albumin/phosphate-buffered saline.

Table 2. Reactivity of monoclonal B532 against cell lines

Cell lines	Sig	EBNA	Ia(243)	HLA(368)	532
B cells					
8392	+	+	+	+	+
Wi-L2	+	+	+	+	+
BJA-B-EBV	+	+	+	+	+
BJA-B	+	-	+	+	-
Loukes	+	-	+	+	-
Ramos	+	-	+	+	-
Lymphoblastoid non-B cells					
RPMI 8402	-	-	+	+	-
CCRF-CEM	-	-	-	+	-
NALL-1	-	-	+	+	-
NALM-6	-	-	+	+	+
HSB-2	-	-	+	+	-
Myeloid cells					
ML-1	-	-	+	+	-
Normal PBLs and EBV-infected cells					
AT - PBL	-	-	-	+	-
AT - PBL-EBV	+	+	+	+	+
L.W. - PBL	-	-	-	+	-
L.W. - PBL-EBV	+	+	+	+	+
K.H. - PBL	-	-	-	+	+
K.H. - PBL-EBV	+	+	+	+	-
B cells*	+	-	+	+	-
T cells*	-	-	-	+	-

EBNA, Epstein-Barr nuclear antigen.

* The T and B cells from a normal PBL were separated from each other by erythrocyte rosetting and were examined independently.

FIG. 1. (Left) Membrane fluorescence of B-cell line RPMI 8392 after staining with monoclonal antibody B532. (Right) Lack of staining in corresponding T-cell line RPMI 8402 with B532. ($\times 460$.)

struments, Westwood, MA). A 5-W argon laser source (488 nm) was used for fluorescence activation.

Neutralization of Cytotoxicity. Cytotoxic T lymphocytes specific for EBV-infected autologous B cells were generated by cocultivation of PBL twice with previously established mitomycin-treated B-cell lines, exactly as described (29). The twice-stimulated T cells were then tested in a standard ^{51}Cr -release assay for cytotoxicity against another aliquot of the same B-cell line.

RESULTS

Binding of B532 to Various Normal and Cultured Cell Lines. The reactivity of the hybridoma supernatant B532 against cultured cell lines (Table 1) was examined by using an indirect

binding assay. High levels of binding occurred with all EBV-infected lymphoblastoid B-cell lines, in particular the B-cell line RPMI 8392, but no binding (i.e., $\text{cpm} \leq 500$) occurred with RPMI 8402, the T-cell line autologous to RPMI 8392. Furthermore, no binding was noted with isolated blood T cells, non-EBV-infected B cells (such as BJA-B and LNPL), myeloid lines, or normal PBLs. The EBV-infected BJA-B and EBV-infected PBL B cells did show substantial binding. The values shown represent the average of triplicate wells.

Further studies on the reactivity of antibody B532 were done by immunofluorescence (Table 2). All cells that were EBV-infected and transformed as indicated by the presence of the Epstein-Barr nuclear antigen showed membrane fluorescence when stained with B532 (Fig. 1). The staining was independent

FIG. 2. Sorter profile of cells stained for reactivity with B532. (A) Lack of detectable staining in a normal donor's PBLs. (B) Fluorescence-positive cells ($\geq 90\%$) in the donor's corresponding EBV-infected lymphoblastoid B-cell line. The dotted lines depict staining with MOPC-21 as a control.

of surface immunoglobulin, Ia, and HLA markers. All PBLs, isolated T cells, and myeloid lines were negative when stained with the same antibody.

Cytofluorographic Analyses. A preparation of normal PBLs and its corresponding EBV-infected lymphoblastoid line from the same donor were stained with B532 and, as a control, with MOPC 21 (an IgG1 mouse myeloma protein with no known antibody specificity). The population of cells in the EBV line that reacted with B532 was substantially higher in percentage

FIG. 3. Analysis by NaDodSO₄/polyacrylamide gel electrophoresis of ¹²⁵I-labeled cell surface antigen immunoprecipitated with B532. (A) Lack of B532 reactive antigen in a preparation from the T-cell line RPMI 8402. (B) Presence of a B532 reactive antigen of M_r 45,000 in a preparation from the parental B-cell line RPMI 8392. (C) Presence of the same antigen in an established EBV-genome-positive B-cell line, Wi-L2.

FIG. 4. Failure of B532 antibody to inhibit ⁵¹Cr release of EBV-infected B cells by autologous cytotoxic T cells. The cytotoxic T cells were generated *in vitro* by two cycles of stimulation of PBLs by a previously established B-cell line. The assay was carried out in medium alone (▲—▲) or in the continuous presence of B532 (■—■) or of a control monoclonal antibody L368 to HLA-framework antigens, with no EBV specificity (○—○). E:T, effector-to-target cell ratio.

and fluorescence intensity than that observed in the PBLs (Fig. 2). MOPC 21 controls showed less than 5% of background staining.

Immunoprecipitation. Monoclonal antibody B532 precipitated a protein from the crude membrane extract of the immunizing line RPMI 8392 and the EBV-infected B-cell line Wi-L2, which gave a sharp band in NaDodSO₄/polyacrylamide gel electrophoresis corresponding to M_r 45,000 (relative mobilities of 0.49 and 0.51, respectively) (Fig. 3). No band was seen from the extract of the T-cell line RPMI 8402.

Neutralization of Cytotoxicity. To test whether the B532 antibody interacts with lymphocyte-defined membrane antigen (LYDMA), the assumed recognition unit in specific T-cell cytotoxicity for EBV-infected B cells (30–32), we treated 1×10^6 ⁵¹Cr-labeled EBV-infected B cells with 0.1 ml of monoclonal antibody B532 or medium (1 hr at 22°C). These cells were then used as targets in the ⁵¹Cr-release assay with autologous cytotoxic T cells. The B532-treated targets lysed as did nontreated targets (Fig. 4), suggesting that B532 does not react with LYDMA.

Neutralization of Infectivity. B532 did not neutralize the ability of the B95-8 strain of EBV to induce transformation of PBL B cells under the conditions tested.

DISCUSSION

We report the development of a monoclonal antibody that reacts with a membrane antigen present on $\geq 95\%$ of EBV-infected B cells but not with non-EBV-infected B cells, T cells, monocytes, or PBLs. The antibody precipitates a molecule of M_r 45,000 on the surface of the EBV-infected B cells.

B cells are known to acquire several new surface antigens after EBV infection (1, 2, 33–37). These include polypeptides with approximate M_r s of 340,000, 250,000, 160,000, and 70,000 found on B-cell lines actively producing virus (1, 2, 31). They have been designated as “early” and “late” membrane antigens (34, 35) in accordance with whether the antigen is expressed before or after viral DNA synthesis. These antigens also can be

found in the virus envelop itself (36, 37). The antigen with which the B532 monoclonal antibody reacts is none of these, both because of its observed M_r and because it has been found regularly on nonproducer EBV-infected B cells. We take the failure of B532 to neutralize virus infectivity, when added to the B cells before the EBV, as tentative evidence that B532 does not block the virus receptor on the B-cell surface. However, an extensive survey of various virus concentrations and B532 concentrations was not carried out.

Whether B532 recognizes the antigen LYDMA is still an uncertainty. LYDMA, thought to be present on all EBV-infected B cells, is the antigen through which immune cytotoxic T cells recognize EBV-infected B cells (30–32). We attempted to test the relationship of B532 to LYDMA by testing its ability to neutralize the cytotoxicity of *in vitro*-generated cytotoxic T cells for autologous EBV-infected B cells. The failure to neutralize makes it seem unlikely that the B532 monoclonal antibody recognizes LYDMA. However, our *in vitro*-generated EBV-specific cytotoxic T cell may not be exactly equivalent to the EBV-specific cytotoxic cell found in the blood of patients with infectious mononucleosis with which LYDMA was first defined. Further, we cannot exclude the possibility that B532 reacts with a portion of the LYDMA molecule unimportant to the T-cell killer function.

Although the studies reported herein show that the antigen with which antibody B532 reacts is associated with EBV-infected but not with noninfected B cells, this does not define the antigen as a product of EBV DNA. It is possible that the B532 antigen is a product of host B-cell DNA, but that this antigen is induced by EBV infection. A monoclonal antibody like B532 was reported recently (38).

We thank Ms. Evelyn Reitz and Ms. Gayle Yamamoto for expert technical assistance and Ms. Shari Brewster for typing this manuscript. This work was supported by Research Grants AM 21175, AM 07144, AM 06188, RR 00833, and RR 05514 and by a grant from the Rheumatic Diseases Research Foundation. C.D.T. is a Fellow of the Arthritis Foundation.

- Thorley-Lawson, D. A. & Geilinger, K. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5307–5311.
- Hoffman, F. J., Lazarowitz, S. G. & Hayward, S. F. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2979–2983.
- Qualtiere, L. F., Chase, R., Vroman, B. & Pearson, G. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 616–620.
- Huang, C. C., Hou, Y., Woods, L. K., Moore, G. E. & Minowada, J. (1974) *J. Natl. Cancer Inst.* **53**, 655–660.
- Kohler, G., Howe, S. C. & Milstein, C. (1976) *Eur. J. Immunol.* **6**, 292–295.
- Galfre, G., Howe, S. C., Milstein, C., Butcher, G. W. & Howard, J. C. (1977) *Nature (London)* **266**, 550–552.
- Nisonoff, A. (1964) *Methods Med. Res.* **10**, 134–141.
- Slaughter, L., Carson, D. A., Jenson, F. C., Holbrook, T. L. & Vaughan, J. H. (1978) *J. Exp. Med.* **148**, 1429–1434.
- Royston, I., Majda, J. A., Baird, S. M., Meserve, B. L. & Griffiths, J. C. (1980) *J. Immunol.* **125**, 725–731.
- Lampson, L. A. & Levy, R. (1980) *J. Immunol.* **125**, 293–299.
- Reedman, B. & Klein, G. (1973) *Int. J. Cancer* **11**, 499–520.
- Levy, J. A., Virolainen, M. & Defendi, V. (1968) *Cancer* **22**, 517–524.
- Menezes, J., Leibold, W., Klein, G. & Clemente, G. (1975) *Bio-medicine* **22**, 276–284.
- Kaplan, J., Schope, T. C. & Peterson, W. D., Jr. (1974) *J. Exp. Med.* **139**, 1070–1076.
- Hiraki, S., Miyoshi, I., Kubonishi, I., Matsuda, Y., Nakayama, T., Kishimoto, H. & Masuji, H. (1977) *Cancer* **40**, 2131–2135.
- Minowada, J., Tsubota, T., Greaves, M. F. & Walters, T. R. (1977) *J. Natl. Cancer Inst.* **59**, 83–87.
- Adams, R. A., Flowers, A. & Davis, B. J. (1968) *Cancer Res.* **28**, 1121–1125.
- Lozzio, C. B. & Lozzio, B. B. (1975) *Blood* **45**, 321–334.
- Minowada, J., Sugawa, K., Trowbridge, I., Kung, P. & Goldstein, G. in *Advances in Malignant Lymphoma: Etiology, Immunology, Pathology, Treatment*, ed. Kaplan, H. S. & Rosenberg, S. A. (Academic, New York) in press.
- Morikawa, S., Tatsumi, E., Baba, M., Harada, T. & Yasuhira, K. (1978) *Int. J. Cancer* **21**, 166–170.
- Minowada, J., Ohnuma, T. & Moore, G. E. (1972) *J. Natl. Cancer Inst.* **49**, 891–895.
- Collins, S. J., Gallo, R. C. & Gallagher, R. E. (1977) *Nature (London)* **270**, 347–349.
- Klein, G., Giovanella, B., Westman, A., Stehlin, J. S. & Mumford, D. (1975) *Intervirology* **5**, 319–334.
- Minowada, J., Oshimura, M., Abe, S., Greaves, M. F., Janossy, G. & Sandberg, A. A. (1978) *Proc. Am. Assoc. Cancer Res.* **19**, 109 (abstr.).
- Hurwitz, R., Hozier, J., LeBien, T., Minowada, J., Gajl-Peczalska, K., Kubonishi, I. & Kersey, J. (1979) *Int. J. Cancer* **23**, 174–180.
- Dillman, R. O., Handley, H. H. & Royston, I. (1981) *Cancer Res.*, in press.
- Chess, L. & Schlossman, S. F. (1976) in *Manual of Clinical Immunology*, eds. Rose, N. R. & Friedman, H. (Am. Soc. Microbiol., Washington, DC) 1st Ed., p. 77.
- Baird, S. M. (1979) *J. Immunol.* **122**, 1389–1395.
- Tsoukas, C. D., Fox, R. I., Slovin, S. F., Carson, D. A., Pellegrino, M., Fong, S., Pasquali, J. L., Ferrone, S., Kung, P. & Vaughan, J. H. (1981) *J. Immunol.* **126**, 1742–1746.
- Svedmyr, E. & Jondal, M. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1622–1626.
- Jondal, M. (1976) *Clin. Exp. Immunol.* **25**, 1–5.
- Rickinson, A. B., Crawford, D. & Epstein, M. A. (1977) *Clin. Exp. Immunol.* **28**, 72–79.
- Thorley-Lawson, D. A. & Edson, C. M. (1979) *J. Virol.* **32**, 458–467.
- Ernberg, I., Klein, G., Kourilsky, F. M. & Silvestre, D. (1974) *J. Natl. Cancer Inst.* **53**, 61–65.
- Sairenji, T., Hinuma, Y., Sekizawa, T. & Yoshida, M. (1977) *J. Gen. Virol.* **38**, 111–120.
- Silvestre, D., Kourilsky, F. M., Klein, G., Yata, Y., Neaupa-Sautes, C. & Levy, J. P. (1971) *Int. J. Cancer* **8**, 222–233.
- North, J. R., Morgan, A. J. & Epstein, M. A. (1980) *Int. J. Cancer* **26**, 231–240.
- Kintner, C. & Sugden, B. (1981) *Nature (London)* **294**, 458–460.