Immunology 1977 **32** 475

Identity of a T-lymphocyte inhibitor with mouse immunoglobulin in the serum of tumour-bearing mice

JULIA G. LEVY, R. McMASTER, BARBARA KELLY, R. B. WHITNEY & D. G. KILBURN
Department of Microbiology, University of British Columbia, Vancouver, Canada

Received 28 *June* 1976; *accepted for publication* 23 *July* 1976

Summary. A component of the serum of tumour-bearing mice has been shown to be inhibitory to the immunological function of normal mouse T cells. This factor fractionates with monomeric immunoglobulin upon gel filtration. Studies were carried out utilizing goat antisera to the major immunoglobulin chains of mouse Ig (κ, γ, α and μ) mixed with the immunoglobulin-rich fraction of serum from normal mice and tumour bearers and passed through immunoadsorbent columns prepared with rabbit anti-goat immunoglobulin. Such studies showed that the inhibitory activity in tumour-bearing serum could be removed after treatment with anti-κ, anti-γ and anti-μ chain antisera but not by treatment with either anti-α chain or goat immunoglobulin. That the inhibitory activity of tumour-bearing immunoglobulin could be attributed to simple quantitative differences in the levels of IgM and IgG in the test samples was discounted by quantification of the amounts of immunoglobulins in normal and tumour-bearing sera.

INTRODUCTION

The serum of both cancer patients and experimental animals bearing transplantable tumours has been shown to contain component(s) which are capable of suppressing the ability of normal lymphocytes to undergo a proliferative *in vitro* response to either mitogenic agents, specific antigens or allogeneic cells (Brooks, Netsky, Normansell & Horwitz, 1972; Sucui-Foca, Buda, McManus, Thiens & Recomtsma, 1973; Nimberg, Glasgow, Menzoian, Constantian, Cooperband, Mannick & Schmid, 1975; Whitney & Levy, 1974; Whitney & Levy, 1975a and b; Levy, Smith, Whitney, McMaster and Kilburn, 1976; Vandenbark, Burger, Finke & Vetto, 1976; Fernbach, Kirchner, Bonnard & Herberman, 1976). In one instance, the immunosuppressive activity was attributed to an immunoglobulin (Brooks *et al.*, 1972); in another, the immunosuppressive component has been partially characterized as a low mol. wt peptide (Nimberg *et al.*, 1975). It has been suggested by others that antigen–antibody complexes may be responsible for non-specific immunosuppression in that such complexes might be capable of binding via their Fc fragments to the surface of lymphocytes bearing Fc receptors, and blocking the functional activity of the affected cells (Gorczynski, Kontiainen, Mitchison & Tigelaar, 1974).

In this laboratory, preliminary attempts have been made to biochemically characterize the serum suppressor found in a variety of strains of mice bearing a variety of tumours. The suppressive component was found to fractionate with 7S immunoglobulins on Sephadex G-150 and G-200, to be stable to fractionation at pH 2·5 on Sephadex G-150,

Correspondence: Dr J. G. Levy, Department of Microbiology, University of British Columbia, 2075 Westbrook Place, Vancouver B.C., Canada V6T 1W5.

and to be removed from serum on immunoadsorbent columns prepared with sheep anti-mouse immunoglobulin (Levy *et al.*, 1976). These data imply the immunoglobulin nature of the inhibitor but the acid fractionation data indicate that an antigen-antibody complex is not involved. Work on the mode of action of this serum inhibitor has shown that it is preferentially suppressive to apparently all T-cell functions, that it is not cytotoxic, and that its effect is essentially immediate and irreversible (Levy *et al.*, 1976; Whitney & Levy, 1975b).

The present study was undertaken in an attempt to determine unequivocally whether or not this serum inhibitor was in fact associated with immunoglobulins in tumour-bearing mice, and if so, which classes or subclasses were involved.

MATERIALS AND METHODS

Experimental animals

DBA/2J and BALB/c mice (Jackson Laboratory, Bar Harbor, Maine) between 2 and 4 months of age were used in all experiments.

Tumours

The tumour system used in this study was a methylcholanthrene induced rhabdomyosarcoma (M-1), obtained originally from the Jackson Laboratory, and maintained in this laboratory both in culture and in DBA/2J mice for the past 4 years. Methods for the maintenance and culture of the tumour have been described elsewhere (Whitney, Levy & Smith, 1974).

The myeloma line used for the preparation of mouse κ chains was the MOPC-46 P4 and was obtained from the Salk Institute. It was maintained as an ascites tumour in BALB/c mice or stored at $-70°$ in dimethyl sulphoxide (DMSO).

Collection and preparation of serum

Blood was taken from both normal and tumour-bearing animals by exsanguination from the heart or by retro-orbital bleedings. Tumour-bearing animals were killed when they had tumours between 3·0 and 4·0 g in weight. Blood was allowed to clot overnight at 4° after which the serum was removed, centrifuged to remove residual erythrocytes, inactivated at 56° for 30 min and stored at $-20°$.

All the work reported herein was carried out on the immunoglobulin-rich fraction of sera from a Sephadex G-150 column. Detailed procedures for this fractionation have been published previously (Levy *et al.*, 1976).

Assay for inhibitory serum factor

Sera and subfractions were assayed as previously described (Whitney *et al.*, 1974; Whitney & Levy, 1974; 1975a). In brief, 5×10^5 normal DBA/2J spleen cells were cultured in 0·20 ml of RPMI-1640 medium supplemented with 2·5 per cent foetal calf serum in plastic microtitre plates (Linbro Chemical Company, New Haven, Connecticut) with various concentrations of the test material from either normal or tumour bearing DBA/2J serum. Concanavalin A (Con A) was added in a volume of 0·05 ml at a concentration of 1 μg/ml. The cultures were incubated for 48 h after which 1·0 μCi (^{3}H)thymidine (New England Nuclear) was added. Cultures were harvested and [^{3}H]thymidine incorporation was measured 18 h later. Values achieved by cultures at equivalent quantities of normal mouse serum were taken as the 100 per cent level and per cent inhibition was calculated by comparing the uptake values of samples containing equivalent quantities of tumour-bearing serum to the 100 per cent value.

Data presented using this assay represents the mean values of at least three replicate cultures for each data point. Standard errors were in all cases less than 10 per cent.

Preparation of immunoadsorbents

An immunoadsorbent of rabbit anti-goat immunoglobulin conjugated to Sepharose 4-B was used. It was prepared according to the method of Porath, Axen & Ernback (1967), and contained 6·0 mg of rabbit immunoglobulin per ml of Sepharose. The binding capacity of the adsorbent was 0·55 mg goat immunoglobulin per ml Sepharose.

An immunoadsorbent containing mouse κ chains was also prepared by the above method; κ chains were prepared by collecting ascites fluid from BALB/c mice bearing the MOPC 46 P4 myeloma. The ascites fluid was precipitated with 40 per cent saturated $(NH_4)_2SO_4$, and chromatographed through DEAE cellulose and Sephadex G-150 to obtain κ chains according to the method described by Heimer, Schwartz, Engle & Woods (1963). The adsorbent contained 12·5 mg protein/ml Sepharose.

Antisera

Anti-κ-chain antibody. Goat anti-mouse Ig was raised using i.m. injections 2 weeks apart of 500 μg of

commercially prepared mouse Ig (Miles). The goat was bled 2 weeks after the last immunization, the serum was inactivated at 56° for 30 min and stored at −20°. The anti-κ-chain antibody was prepared by passing the antisera over the immunoadsorbent prepared with MOPC-46 P4-derived κ chains. The adsorbed antibody was eluted with Sorensen's buffer (pH 2·5), neutralized, and concentrated by precipitation with 40 per cent saturated $(NH_4)_2SO_4$ to a final concentration of 2·0 mg per ml.

Anti-μ-chain antibody. Goat antisera to mouse μ chains was obtained from Bionetics (Kensington, Maryland, lot no. 231–73-4) and contained 12·1 mg protein per ml.

Anti-α-chain antibody. Goat antisera to mouse α-chains was obtained from Bionetics (Kensington, Maryland, lot no. 231-73-1) and contained 14·7 mg protein per ml.

Anti-7S globulins antiserum. Goat antisera to mouse 7S globulins (essentially γ specific) was obtained from Bionetics (Kensington, Maryland, lot no. 231-71-9) and contained 14·1 mg protein per ml. None of the antisera obtained from Bionetics contained any detectable anti-κ chain activity.

Anti-mouse IgG. Goat anti-mouse IgG was obtained from Cappel Laboratories (Downington, Philadelphia, lot no. 8726) and contained 24·0 mg protein per ml. This antiserum contained both anti-γ- and κ-chain activity.

Goat immunoglobulin. Goat Ig was obtained from Miles and contained 15 mg protein per ml.

Rabbit anti-goat Ig antisera. Adsorbent-purified goat anti-mouse Ig was used to immunize a rabbit to goat Ig. 1·0 mg of goat Ig was administered in 50 per cent complete Freund's adjuvant twice at 2-week intervals. Serum was collected 2 weeks after the last injection, heat inactivated at 56° for 30 min and stored at −20°.

Immunoadsorbent experiments

Aliquots of both normal and tumour-bearing Ig-rich material were mixed at concentrations of 1·5 mg protein with the following concentrations of the various antisera: purified anti-κ chain, 0·75 mg; anti-μ chain, 2·5 mg; anti-α chain, 3·0 mg; anti-7S Ig, 3·0 mg; anti-mouse IgG, 4·6 mg; goat immunoglobulin, 3·0 mg. These mixtures were allowed to react for 18 h at 4°. Immunoadsorbent columns containing 6·0 ml of Sepharose 4 B linked to rabbit anti-goat Ig were prepared and washed with RPMI 1640. The mixture containing the mouse Ig rich fraction and the various goat sera (in volumes not exceeding 0·5 ml) were applied to and allowed to penetrate individual columns, and were washed in with further 0·5 ml of RPMI 1640. Adsorption was allowed to take place over a period of 30 min after which the columns were washed with 1640 and 1·0 ml eluted fractions were collected and read for 280 nm absorbance on a DBG spectrophotometer. The individual fractions from each adsorption were sterilized by filtration and assayed for inhibitory activity using Con A stimulated DBA/2J spleen cells in culture as described above. Three different concentrations of each fraction were tested in culture and each test was run in triplicate. The per cent inhibition attributed to tumour-bearing serum was calculated by comparing the Con A responses of cells in the presence of tumour-bearing serum fractions to the equivalent fraction and protein concentration of normal mouse serum. This experiment was performed on five different occasions.

Quantitation of mouse immunoglobulin

The amount of total mouse immunogobulin in each fraction was quantitated by radial immunodiffusion. Radial immunodiffusion plates were prepared by adding 2 per cent ionagar, anti-mouse Ig serum to a final concentration of 5 per cent. 10-μl aliquots of various dilutions of mouse serum fractions were added to punched-out wells and the plates were kept at 4° for 24 h.

RESULTS

Assay of Ig-rich fractions of mouse sera

Serum taken from normal and tumour-bearing DBA/2J mice was fractionated on Sephadex G-150, precipitated and reconstituted and dialysed in 0·85 per cent NaCl. The amount of protein in both the normal (NF_1) and tumour-bearing (TF_1) fraction was adjusted to 7·5 mg protein per ml. The fractions were assayed for their inhibitory activity by culturing Con A-stimulated normal DBA/2J spleen cells in the presence of varying concentrations of TF_1 and NF_1. A typical set of data are shown in Table 1. As has been previously noted (Whitney & Levy, 1974), at higher concentrations, the serum fraction from normal mice was also somewhat inhibitory.

Table 1. Assay for inhibitory activity in the Ig-rich fraction of tumour-bearing serum. Inhibition was quantified as the percentage decrease in [^{3}H]thymidine uptake in tumour-bearing fractions (TF_1) in comparison to equivalent levels of normal serum fractions (NF_1)

Protein concentration (mg/ml in culture)	Incorporation of [^{3}H]thymidine (CPM) in Ig-rich fractions		Percentage inhibition
	NF_1	TF_1	
0·3	202,205	81,726	59·6
0·6	110,775	2030	81·7
0·9	46,421	700	98·5

Characterization of the Inhibitory factor

Goat antiserum prepared against various mouse Ig chains (anti-κ chain, anti-μ chain, anti-α chain and anti-γ chain) were mixed with TF_1 and NF_1 and subsequently passed through immunoadsorbent columns prepared with anti-goat Ig. The material which was not absorbed was titrated for the presence of inhibitor. All experiments were run using a control of goat-Ig with TF_1 and NF_1 adjusted to quantities equivalent to the amounts of goat antiserum used. Initial experiments using goat anti-κ chain indicated that this anti-serum when incubated with TF_1 and NF_1 successfully removed essentially all of the inhibitory material from TF_1 when passed through the immunoadsorbent. Further experiments were run with goat antisera to the three major classes of heavy chains, always concurrently with goat Ig as a negative control and anti-κ chain as

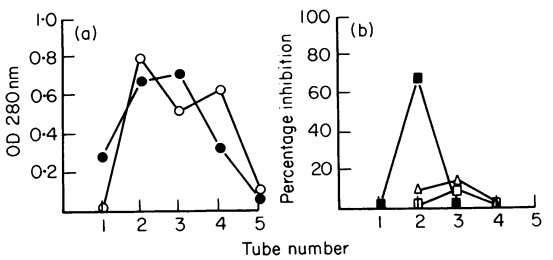

Figure 2. (a) Elution profile of TF_1 and NF_1 after mixture with goat anti-μ chain antiserum and passage through an immunoadsorbent containing anti-goat Ig. (○——○) NF_1; (●——●) TF_1. (b) Relative degrees of inhibition of Con A responses attributable to individual tubes eluted. Each tube is compared to the equivalent protein concentration and fraction number from NF_1. (△——△) anti-μ chain treatment; (■——■) control using goat Ig; (□——□) positive control using anti-κ chain.

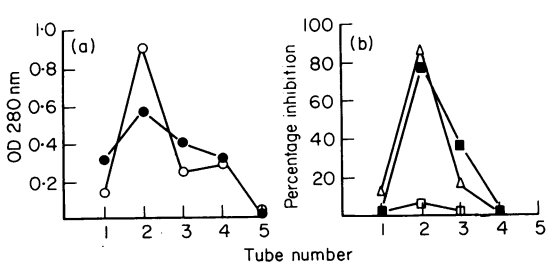

Figure 1. (a) Elution profile of TF_1 and NF_1 after mixture with goat anti-α chain antiserum and passage through an immunoadsorbent containing anti-goat Ig (○——○). NF_1; (●——●) TF_1. (b) Relative degrees of inhibition of Con A responses attributable to individual tubes eluted. Each tube is compared to the equivalent protein concentration and fraction number from NF_1. (△——△) Anti-α chain treatment; (■——■) control using goat Ig; (□——□) positive control using anti-κ chain.

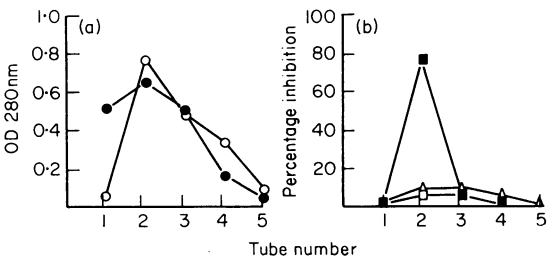

Figure 3. (a) Elution profile of TF_1 and NF_1 after mixture with goat anti-γ chain antiserum and passage through an immunoadsorbent containing anti-goat Ig. (○——○) NF_1; (●——●) TF_1. (b) Relative degrees of inhibition of Con A response attributable to individual tubes eluted. Each tube is compared to the equivalent protein concentration and fraction number from NF_1. (△——△) anti-γ chain; (■——■) control using goat Ig; (□——□) positive control using anti-κ chain.

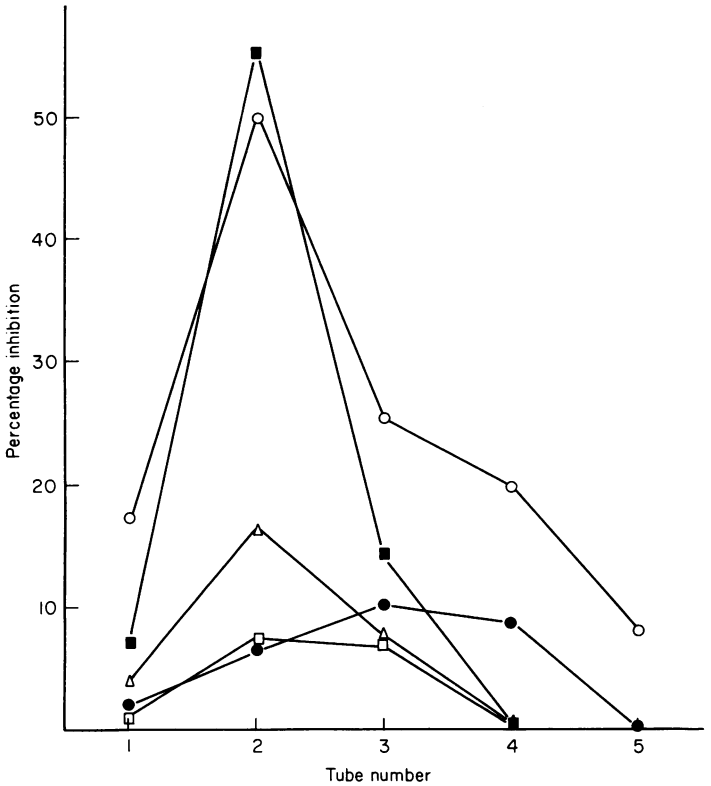

Figure 4. Averaged results from five individual experiments using all the test goat antisera, and titration of the relative degrees of inhibition of the Con A response of normal lymphocytes. (○——○) anti-α chain; (●——●) anti-μ chain; (△——△) anti-γ chain; (□——□) positive control with anti-κ chain; (■——■) negative control with goat Ig.

a positive control. Representative results from such experiments are shown in Figs 1, 2 and 3, showing the elution profile of the unabsorbed materials coming off the immunoadsorbent columns and the relative degrees of inhibition from each fraction in comparison to results obtained with anti-κ chain antiserum and goat Ig.

As can be seen from the figures both the anti-γ chain and anti-μ chain antisera removed inhibitory activity from TF_1, while anti-α chain antiserum did not. Fig. 4 shows a composite graph of an average of five different experiments run with the various goat antisera and goat Ig.

These data show that the inhibitory factor found in the serum of tumour-bearing animals shares identity with mouse κ, γ and μ chains. Because it was possible that immunoglobulins as such were inhibitory to lymphocyte transformation, the Ig content of the serum fractions used here was estimated using radial immunodiffusion. In no instance was the Ig content of TF_1 higher than that in the NF_1 samples tested. In fact, the Ig content of TF_1 samples consistently were lower than comparable NF_1 samples.

It was concluded that the inhibitory factor found in the serum of tumour-bearing mice showed positive identity with both IgG and IgM but that this was some specific type of immunoglobulin since the Ig content of the tumour-bearing serum was never higher than that found in normal mouse serum.

DISCUSSION

Previous experiments in this laboratory have shown that the Ig fraction of serum from tumour-bearing mice contains a component which appears to inhibit

a variety of immune reactions of normal lymphocytes including, stimulation of lymphocyte proliferation by Con A, the proliferative response to specific antigens, the mixed lymphocyte reaction, and the *in vitro* development of plaque-forming cells to sheep red blood cells. The observation that neither the lipopolysaccharide proliferative response, nor the development of plaque formers to T-cell independent antigens were inhibited led to the conclusion that the inhibitory activity of the serum component was directed mainly toward T cells (Levy *et al.*, 1976). The previous study also indicated that the inhibitor could be removed from the serum by passage of sera through an immunoadsorbent prepared with goat anti-mouse Ig, that it had a mol. wt of about 150,000 and that it was not inactivated or broken into smaller components by treatment and gel filtration at pH 2.

The present study constitutes an attempt to further characterize this inhibitor. The somewhat indirect immunoadsorbent procedure used here was developed for two important reasons. Firstly, in order to test a variety of antisera for their ability to inactivate or remove the inhibitor, it was essential to use a carefully standardized procedure so that each sample was comparable to other samples. For this reason, the various goat antisera-mouse sera mixtures were all passed through the same immunoadsorbent which was known to be capable of removing all of the goat Ig present in the mixtures. Secondly, experimental work carried out in this laboratory (Kilburn, Fairhurst, Levy & Whitney, 1976) has shown that the inhibitory activity of the tumour-bearing serum is enhanced by the presence of immune complexes. Thus it was essential to utilize a technique which would remove such complexes.

The results indicate clearly that the inhibitory component shares immunological identity with κ, γ and μ chains. That this activity was not due simply to a quantitative difference in the levels of Ig in TF_1 and NF_1 was established by Ig quantification using radial immunodiffusion. There are a few possible explanations for these observations. There may be immunoregulatory immunoglobulins occurring in the serum of tumour-bearing animals at higher levels than those found in normal animals which may have specific, as yet unknown characteristics. Even though our previous studies have indicated that this inhibitory component is not a classical immune complex by virtue of its mol. wt and its stability under acidic conditions (Levy *et al.*, 1976), it is still possible that the inhibitory immunoglobulin is comprised of an immunoglobulin molecule associated with some other, presumably relatively low mol. wt component, which has the capacity to alter the properties of the normal Ig. We are aware that many of the inhibitory properties of this component are closely analogous to those attributed to the C-reactive protein (CRP) found in the serum of humans with a variety of inflammatory conditions (Mortensen & Gewurz, 1976). This material, which has a mol. wt of approximately 120,000, has no immunological cross-reactivity with Ig and is composed of six non-covalently linked monomers with mol. wt of about 20,000 (Gotschlich & Edelman, 1965; Gotschlich & Edelman, 1967). The possibility that an analogous molecule could be associated with immunoglobulin, and thereby render its properties altered with respect to its interaction with T cells is currently being investigated.

ACKNOWLEDGMENT

This work was supported by grant number 65-6048 from the National Cancer Institute of Canada.

REFERENCES

Brooks W.H., Netsky H.G., Normansell D.E. & Horwitz D.A. (1972) Depressed cell-mediated immunity in patients with primary intracranial tumors. *J. exp. Med.* **136**, 1631.

Fernbach B.R., Kirchner H., Bonnard G.D. & Herberman R.B. (1976) Suppression of mixed lymphocyte response in mice bearing primary tumors induced by murine sarcoma virus. *Transplantation*, **21**, 381.

Gorczynski R., Kontiainen S., Mitchison N.A. & Tigelaar R.E. (1974) Cellular selection and regulation in the immune response (ed. by G.M. Edelman), p. 143. Raven Press, New York.

Gotschlich E.C. & Edelman G.M. (1965) C-reactive protein: a molecule composed of subunits. *Proc. natl. Acad. Sci. (Wash.)*, **54**, 558.

Gotschlich E.C. & Edelman G.M. (1967) Binding properties and specificity of C-reactive proteins. *Proc. natl. Acad. Sci. (Wash.)*, **57**, 706.

Heimer R., Schwartz E.R., Engle, Jr, R.L. & Woods K.R. (1963) Relationship of structure to the thermal solubility characteristics of a Bence-Jones protein. *Biochemistry*, **2**, 1380.

Kilburn D.G., Fairhurst M., Levy J.G. & Whitney R.B. (1976) Synergism between immune complexes and serum from tumor bearing mice in the suppression of mitogen responses. *J. Immunol.* (In press.)

LEVY J.G., SMITH A.G., WHITNEY R.B., MCMASTER R. & KILBURN D.G. (1976) Characterization of a T-lymphocyte inhibitor in the serum of tumour-bearing mice. *Immunology*, **30**, 565.

MORTENSEN R.F. & GEWURZ H. (1976) Effects of C-reactive protein on the lymphoid system. *J. Immunol.* **116**, 1244.

NIMBERG R.B., GLASGOW A.H., MENZOIAN J.O., CONSTANTIAN M.B., COOPERBAND S.R., MANNICK J.A. & SCHMID K. (1975) Isolation of an immunosuppressive peptide fraction from the serum of cancer patients. *Cancer Res.* **35**, 1489.

PORATH J., AXEN R. & ERNBACK S. (1967) Chemical coupling of proteins to agarose. *Nature (Lond.)*, **215**, 1491.

SUCUI-FOCA N., BUDA J., MCMANUS J., THIENS J. & RECOMTSMA K. (1973) Impaired responsiveness of lymphocytes and serum inhibitory factors in patients with cancer. *Cancer Res.* **33**, 2373.

VANDENBARK A.A., BURGER D.R., FINKE P. & VETTO R.M. (1976) The effects of plasma from guinea pigs with tumor on PHA stimulated lymphocyte cultures. *Cell Immunol.* **22**, 334.

WHITNEY R.B. & LEVY J.G. (1974) Suppression of mitogen responses by serum from tumour-bearing mice. *Europ. J. Cancer*, **10**, 739.

WHITNEY R.B. & LEVY J.G. (1975a) Effects of sera from tumor-bearing mice on mitogen and allogeneic cell stimulation of normal lymphoid cells. *J. natl. Cancer Inst.* **54**, 233.

WHITNEY R.B. & LEVY J.G. (1975b) Mode of action of immunosuppressive substances in sera of tumor-bearing mice. *J. natl. Cancer Inst.* **55**, 1447.

WHITNEY R.B., LEVY J.G. & SMITH A.G. (1974) Influence of tumor size and surgical resection on cell-mediated immunity in mice. *J. natl. Cancer Inst.* **53**, 111.