Clin. exp. Immunol. (1974) **18,** 177–186.

ROSETTE FORMATION BY MOUSE LYMPHOCYTES

II. T-CELL SPECIFICITIES IN A CRL SUBPOPULATION

A. ARNAIZ-VILLENA, MARIA I. C. GYÖNGYÖSSY AND J. H. L. PLAYFAIR

Department of Immunology, Middlesex Hospital Medical School, London

(*Received* 4 *January* 1974)

SUMMARY

Mouse spleen and lymph node lymphocytes with receptors for complement (CRL) were prepared by a standard rosetting method and the identity of the central lymphocyte studied by indirect immunofluorescence with heterologous antisera specific for immunoglobulin and for T cells. About 30% of CRL were shown to be T cells and the remainder immunoglobulin-bearing (B) cells.

In thymus-less 'nude' mice a population of immunoglobulin-negative, non-T cell CRL was found.

INTRODUCTION

It has been shown that receptors exist on lymphocytes, macrophages, granulocytes and platelets for modified C3 (C3b) in several mammalian species (Uhr, 1965; Uhr & Phillips, 1966; Bianco, Patrick & Nussenzweig, 1970; Huber *et al.*, 1968; Henson, 1969; Eden, Bianco & Nussenzweig, 1973). Receptors for C3 inactivator-cleaved C3b have been also described on lymphocytes (Ross *et al.*, 1973b). The lymphocytes bearing complement receptors (CRL) are generally considered to be part of the immunoglobulin (Ig) bearing (B) lymphocyte population (Bianco *et al.*, 1970; Nussenzweig *et al.*, 1971). Studies in the mouse, using selective depletion of θ-bearing (T) cells and of the CRL-rosetting population by means of cytotoxicity and sedimentation velocity respectively, followed by statistical analysis, suggested that θ-bearing cells and CRL were non-overlapping populations and that CRL and Ig-bearing cells were highly coincident (Bianco & Nussenzweig, 1971). Specific isolation of a small fraction (15–30%) of the CRL was obtained with about 95% purity (Eden, Bianco & Nussenzweig, 1971); in this recovered population, no cells were θ-bearing (T) cells and the majority were Ig-positive (B) cells. It was also shown that a number of CRL can regenerate in lymph node from bone marrow in the absence of thymus (Nussenzweig *et al.*, 1971) and that CRL localize preferentially in the lymph node and spleen B areas (Dukor, Bianco & Nussenzweig, 1970; Silveira, Mendes & Tolnai, 1972).

Correspondence: Dr Antonio Arnaiz-Villena, Department of Immunology, Middlesex Hospital, 30–40 Tottenham Street, London W1P 9PG.

However, the demonstration of a lymphocyte subpopulation which was CR-positive and Ig-negative in mouse spleen (10–20%) (Ross *et al.*, 1973a), taken together with the low yields of the cytotoxic and depletion methods, led us to the direct study of T specificities in CRL, combining the standard test for forming CRL rosettes with indirect immunofluorescence.

MATERIALS AND METHODS

Animals

(C57Bl × BALB/c) F_1 mice were bred in our own laboratory from inbred parental strain mice (Laboratory Animals Centre, Carshalton, Surrey) (Playfair, 1968). Male mice were used in all the experiments. This particular strain was chosen because the antisera described below had been standardized in it, and also because of the low incidence of spontaneous

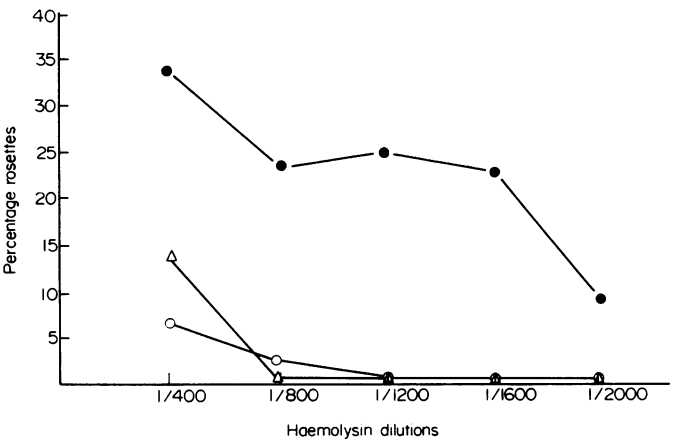

FIG. 1. Titration of antibody concentration and number of clusters formed by spleen cells and EA (○), EACi (△) and EAC (●).

pathology encountered in these mice. In some experiments, non inbred nu-nu 'nude' mice were used (MRC, Laboratory Animals Centre, Carshalton, Surrey).

Preparation of EAC

A standard technique (Bianco *et al.*, 1970) with slight modifications (Arnaiz-Villena, 1973) was used. SRBC (E) (Burroughs Wellcome) were used up to 3 weeks old. Rabbit anti-SRBC antiserum (A) was also obtained from Burroughs Wellcome and inactivated before use at 56°C for 30 min. Anaesthetized male mice were bled from the axillary vessels, blood being kept at 4°C to separate the serum. Aliquots of pooled serum were stored at −20°C and used as the source of complement (C).

The sensitization of E with amboceptor (A) and mouse C was performed as follows. A 5% suspension of washed E and a 1 : 1250 dilution of amboceptor (A) in veronal buffer solution (VBS) were incubated at 37°C for 30 min. This particular dilution (three times the haemagglutination working strength) was selected from a titration curve (Fig. 1). Equal volumes of thrice washed 5% EA and a 1 : 20 dilution of mouse C in VBS were incubated at 37°C for 30 min. Under these conditions very little haemolysis occurred. After three more washes in VBS a final suspension of EAC was made up in either HBSS (Burroughs Wellcome)

or 10 mM EDTA (BDH Chemicals Ltd) HBSS, which inhibits the binding of EAC by PMN, macrophages and monocytes (Lay & Nussenzweig, 1968; Huber & Douglas, 1970).

Cell suspensions

Inguinal, mesenteric and axillary lymph nodes, spleen and thymus cells were obtained by teasing in HBSS at 4°C, aggregates were allowed to sediment for 5 min, and the supernatant was collected. The cells were washed twice at 200 *g* for 5 min at 4°C and resuspended in HBSS at a concentration of 10×10^6 cells per ml. In some cases, phagocytic cells were removed with iron carbonyl (3 mg per 10^8 cells) and a magnet.

Assay for CRL

Disposable, flat-bottomed plastic tubes with caps (45 × 10 mm) were used. 0·5 ml of the HBSS–EAC or of the 10mM EDTA HBSS–EAC suspensions were mixed with 0·5 ml of spleen or lymph node cell suspensions. The tubes were placed at 37°C in a rotor with a horizontal axis and a speed of 60 rev/min. Total leucocytes and total rosettes were counted in the same field after adding one drop of 0·01% Acridine Orange to each tube, under a Leitz epifluorescence microscope, using simultaneous fluorescent and normal transmitted light. By these means it was possible to distinguish the viable cells, the nucleated cells in the middle of the rosettes, and the exact shape of the nucleus in the rosette-forming cell.

At least 200 cells were counted. As controls, suspensions of E, EA and EACi (Ci = inactivated complement) were used instead of EAC (Fig. 1).

Indirect immunofluorescence and antisera

Rabbit anti-mouse IgM, rabbit anti-mouse light chains and rabbit anti-mouse BAθ (Gyöngyössy & Playfair, 1973) were used for indirect immunofluorescence, followed by a FITC-congugated goat anti-rabbit Ig serum. All sera were centrifuged for 5 min at 14,000 *g* in a microcentrifuge before use. For cytotoxicity, unabsorbed anti-θC3H and anti-BAθ, absorbed as previously described, were used (Gyöngyössy & Playfair, 1973) in conjunction with preserved guinea-pig serum (Burroughs Wellcome), absorbed with agarose, as a source of complement.

Fixation and staining

Mouse spleen and lymph node cells or CRL-rosette preparations were immediately fixed with 0·6% (final concentration) glutaraldehyde as described by Gyöngyössy & Playfair (1974), and subsequently stained for IgM, light chains or BAθ. Finally, one drop of mounting medium was added to the pellet and the results recorded under a Leitz incident ultraviolet microscope, 200 or more cells being counted.

Cytotoxicity

Spleen and lymph node suspensions were prepared at a concentration of 2×10^7 per ml. Aliquots of 0·05 ml were washed and the appropriate dilution of the antiserum added to the pellet, followed by incubation for 30 min at 37°C. After washing, complement was added and cytotoxicity assayed by Trypan Blue dye exclusion test (Gyöngyössy & Playfair, 1973).

Absorptions

In order to ensure the specificity of the rabbit anti-BAθ staining, the anti-BAθ antiserum at a dilution of 1 : 4 was mixed with equal volumes of either mouse brain homogenate in

HBSS, mouse thymus cells or nude mouse spleen cells. Unless specified, all of these absorptions were carried out on a tube roller at 4°C overnight.

Phagocytic cells

It has been reported that uptake of Neutral Red is a marker for functional phagocytic cells (Cohn & Wiener, 1963). CRL preparations (10^7 per ml) were made up in 10% foetal calf serum HBSS and one volume of 0·1% Neutral Red PBS was added to nineteen volumes of the CRL suspension. Incubation was carried out at 37°C for 30 min, after which they were washed twice at 100 *g* for 5 min at 4°C in HBSS and examined under the normal light microscope.

RESULTS

Detection of CRL rosettes (*Fig.* 1)

Using E for the standard assay, no rosettes were observed; when EA was used instead of EAC, a small percentage of clusters was obtained at agglutinating doses (erythrocytes and

TABLE 1. Effect on CRL counts of the presence of 10 mM EDTA in the incubation medium*

Cells	Number of experiments	EDTA present (% CRL)	No EDTA present (% CRL)
Lymph node	10	15±2	19±1·7
Spleen	10	24±3·6	34±5

* The values shown are the mean ± one s.d.

erythrocytes plus lymphocytes). In Fig. 1 a plateau can be seen, corresponding to subagglutinating doses of haemolysin in which no EA rosettes are obtained. When EACi (the source of complement being inactivated) was used as indicator, the results were similar to those obtained with EA. With EAC at subagglutinating doses of A, a plateau is reached where only CRL are detected.

The effect of EDTA (*Table* 1)

In EDTA-free medium 34% of spleen cells were CRL, falling to 24% in 10 mM EDTA. This suggests that about 10% of spleen cells carry Mg^{++}-sensitive complement receptors. The CRL population in lymph node is less sensitive to EDTA, reflecting the relative purity of lymphoid cells in this population. By Acridine Orange staining, 90% were viable cells and it is noteworthy that all cells forming rosettes in the presence of EDTA were mononuclear cells of typical lymphoid appearance.

Combined indirect immunofluorescence and standard test for detection of CRL rosettes (*Table* 2)

The rosetting test was performed throughout in the presence of 10 mM EDTA using whole cell suspensions. The immunofluorescent staining for IgM, light chains and BAθ was calculated from duplicate counts with respect to both CRL (rosettes) and non-rosetting cells.

TABLE 2. Percentages of CRL, stained CRL and stained lymphocytes for IgM, LC and BAθ using indirect immunofluorescence

Source	Number of experiments	Percentage of CRL (mean ± s.d.)	Percentage of CRL rosettes staining (mean ± s.d.)			Percentage of non-rosetting cells staining (mean ± s.d.)		
			IgM	LC	BAθ	IgM	LC	BAθ
Normal spleen	10*	22 ± 1·9	59 ± 8·1	76 ± 12	35 ± 6·3	41 ± 2·9	50 ± 15	28 ± 5·4
Normal lymph node	6*	18 ± 2	61 ± 6·8	65 ± 18	30 ± 7	21 ± 6	24 ± 0·5	36 ± 4
Nu/nu spleen	3†	38·6 ± 4·3	n.t.	79 ± 9	3·8 ± 2	n.t.	76 ± 3	3·4 ± 1
Nu/nu lymph node	2†	43 ± 8	n.t.	85 ± 8	0	n.t.	n.t.	1

n.t. = Not tested.

* Pooled results of separate experiments. In each one were pooled spleen or lymph nodes of four different mice.

† Pooled results. Each experiment comprises at least two mice.

For the CRL rosettes, the light chain and BAθ percentages are approximately complementary, adding up to about 100%. In the non-CRL lymph node population however, an unexpectedly high proportion of cells was negative for both B- and T-cell markers. Presumably these include macrophages, monocytes, other myeloid cells and Ig-negative B lymphocytes (Ross *et al.*, 1973a).

Homozygous 'nude' mice show an increase in CRL percentages, but a virtual absence of T-CRL and a population of non-T-non Ig-CRL.

Specificity of the anti BAθ antiserum

The rabbit anti-BAθ antiserum has already been established as highly specific for T cells (Gyöngyössy & Playfair, 1973). However, further absorptions were considered advisable in

TABLE 3. Anti-BAθ staining following absorptions of the antiserum

Tissue used for absorption	Number of absorptions	Percentage of CRL rosettes stained		Percentage of lymphocytes stained	
		Lymph node	Spleen	Lymph node	Spleen
None	None	29	31	32	27
Thymus cells	1	10	16	n.t.	n.t.
Thymus cells	2	0	0	3	0·5
Brain homogenate	1	0	0	1·7	0
Nu/nu mice spleen cells	1	32	30	29	33

Each value is the average of three separate experiments (see Materials and Methods section).

n.t. = Not tested.

view of the unexpected finding of T-CRL (Table 3). Thymus cells and brain homogenate absorbed out the ability to stain T-CRL completely, whereas 'nude' mouse spleen cells did not.

Pretreatment of the cell suspensions with antisera cytotoxic for T cells (Table 4)

Treatment with either anti-BAθ or anti-θC3H serum and complement caused a relative increase in total CRL and a relative decrease of T-CRL compared to the controls. This may indicate that most of the CRL are B cells and that specific depletion of T cells increases the percentage of B-CRL. The incomplete elimination of T-CRL is probably due to the fact that indirect immunofluorescence is more sensitive than cytotoxicity, our cytotoxic antisera being incapable of eliminating all the θ-bearing cells.

Phagocytic cells

As Table 5 shows, none of the cells forming EAC rosettes in the presence of EDTA took up Neutral Red, suggesting that they are in fact lymphocytes. CRL counts were the same before and after Neutral Red incubation.

TABLE 4. CRL numbers and anti-BAθ immunofluorescent staining after pretreatment of spleen cells with antisera cytotoxic for T cells*

Pretreatment	Percentage of cells killed	Percentage of CRL	Percentage of CRL stained with anti-BAθ
Anti-BAθ+C	30	30	18
Normal rabbit serum+C	5	18	27
Anti-θC3H+C	24	22	10
Normal AKR serum+C	9	16	26

The viability of the starting populations was 95%.

* Each value is the average of two separate experiments (see Materials and Methods section).

TABLE 5. Identification of phagocytic and non-phagocytic cells by means of Neutral Red uptake*

Cell population	Percentage uptake of N.R. by cells	Percentage uptake of N.R. by CRL rosettes	Percentage of CRL rosettes stained with anti-BAθ
Lymph node			
Untreated	6	5	n.t.
EDTA I.C.†	0·8	0	32
EDTA	n.t.	0	30
Spleen			
Untreated	12	20	21
EDTA I.C.	2·5	0	23
EDTA	n.t.	0	25

n.t. = Not tested.

* Each value is the average of three separate experiments (see Materials and Methods section).

† I.C. = carbonyl iron powder.

DISCUSSION

Using the technique of glutaraldehyde fixation and immunofluorescence staining of rosettes described by Gyöngyössy & Playfair (1974), we have found that, contrary to the normal view, some 30% of CRL in the mouse lymph node and spleen are T cells (T-CRL). This population may well be identical to the Ig-negative CRL population reported by others (Ross *et al.*, 1973a). An important advantage of the present technique is the use of whole cell populations not subjected to any cell depletion; methods such as CRL-rosette isolation, removal of phagocytic cells, etc., are invariably associated with some non-specific cell loss, which could

be critical when minority populations are concerned. On a rough estimate based on our figures about 20% of spleen T cells, and 10% of lymph node T cells are CRL.

The technique used for detection of CRL was that used by Bianco *et al.* (1970), Dukor *et al.* (1970) and Pincus, Bianco & Nussenzweig (1972). Like them and others (Michlmays & Huber, 1970; Jondal, Holm & Wigzell, 1972), we used subagglutinating doses of whole rabbit anti-SRBC antiserum rather than a 19S fraction. The essentially negative rosette counts, in the absence of complement (EA, EACi) (Fig. 1) are no doubt due to the low concentration of antibody employed. With higher concentrations, EA rosettes are obtained (Soteriades-Vlachos, Gyöngyössy & Playfair, 1974).

Our experiments with EDTA confirm the existence of two types of receptor for complement on the surface of cells, one Mg^{++}-dependent (on monocytes, polymorphs and macrophages), and one not Mg^{++}-dependent, and apparently restricted to lymphocytes. In the presence of EDTA (Table 1), a fall in CRL rosette counts was observed in both lymph node and spleen cells. This fall corresponded to the disappearance of the non-lymphoid rosettes, as judged by: (a) the failure of the remaining CRL to take up Neutral Red; (b) the uniformly mononuclear appearance of all the remaining CRL when stained with Acridine Orange; (c) the finding that complement rosettes made by cells from 'nude' mouse spleen after removal of B cells and phagocytic cells, and non-lymphoid as judged by electron microscopy, are all EDTA-sensitive (Arnaiz-Villena, Hudson & Penfold, unpublished data). We therefore feel justified in referring to the EDTA-resistant CRL as 'lymphoid'.

In normal mice (Table 2), about 30% of the lymphoid CRL population in both spleen and lymph node appear to be T cells (T-CRL), whilst about 70% carry surface Ig (B-CRL). It seems likely, though we have not proved this, that these are two separate populations, in which case virtually all the CRL are accounted for. In the 'nude' mouse, however, there is a deficit, 15–20% of lymphoid CRL being Ig-negative by fluorescence. It is an interesting possibility that these include the T-cell precursors postulated by Komuro & Boyse (1973) and shown to develop T-cell specifities under the influence of the thymus hormone or thymus epithelium (Wortis, Nehlsen & Owen, 1971; Loor & Kindred, 1973). Preliminary experiments using T- and B-cell populations purified by separation on biodegradable columns (Schlossmann & Hudson, 1973) have also shown that a number of CRL are T cells (Hudson & Arnaiz-Villena, unpublished data).

Our results are in apparent conflict with previous reports showing that θ-bearing (T) cells and CRL are non-overlapping populations (Bianco & Nussenzweig, 1971), in which the approach has been based on either B-cell depletion or anti-θ cytotoxicity combined with statistical analysis, or on the release of CRL by dissociation of the rosettes (Eden *et al.*, 1971). In the latter case, the very low yield (15–30% of the original CRL) makes it quite possible that the T-CRL were lost, while in the former case it cannot be excluded on purely statistical grounds that some 20% of the cells were CRL. In support of this idea is the finding that the absolute number of CRL in lymph nodes is reduced by thymectomy (Nussenzweig *et al.*, 1971).

In view of the fact that complement and Fc receptors (Soteriades-Vlachos *et al.*, 1974) are present on subpopulations of both B and T cells, one cannot help wondering whether the binding of immune complexes may play a part in some T-cell functions; in particular a role for complement in T cell–B cell co-operation (Bitter-Suermann *et al.*, 1973) seems a distinct possibility. It is hoped that experiments on the effect of CRL depletion from T and/or B cells will clarify this point.

ACKNOWLEDGMENTS

Antonio Arnaiz-Villena was in receipt of a British Council-Canada Blanch grant. The work was also supported by the Arthritis and Rheumatism Council, the Medical Research Council, and the World Health Organisation.

REFERENCES

ARNAIZ-VILLENA, A. (1973) *Problaciones linfociticas en el hombre.* Doctoral thesis, Madrid.

BIANCO, C. & NUSSENZWEIG, V. (1971) Theta-bearing and complement receptor lymphocytes are distinct populations of cells. *Science*, **173,** 154.

BIANCO, C., PATRICK, R. & NUSSENZWEIG, V. (1970) A population of lymphocytes bearing a membrane receptor for antigen–antibody–complement complexes. I. Separation and characterization. *J. exp. Med.* **132,** 702.

BITTER-SUERMANN, D., DUKOR, P., GISLER, R.H., SCHUMANN, G., DIERICH, M., KONIG, W. & HADDING, U. (1973) C3 dependence of B-cell activation? *J. Immunol.* **111,** 301.

COHN, Z.A. & WIENER, E. (1963) The particulate hydrolases of macrophages. I. Comparative enzymology, isolation and properties. *J. exp. Med.* **118,** 991.

DUKOR, P., BIANCO, C. & NUSSENZWEIG, V. (1970) Tissue localization of lymphocytes bearing a membrane receptor for antigen–antibody–complement complexes. *Proc. nat. Acad. Sci. (Wash.),* **67,** 991.

EDEN, A., BIANCO, C. & NUSSENZWEIG, V. (1971) A population of lymphocytes bearing a membrane receptor for antigen–antibody–complement complexes. II. Specific isolation. *Cell. Immunol.* **2,** 658.

EDEN, A., BIANCO, C. & NUSSENZWEIG, V. (1973) Mechanism of binding of soluble immune complexes to lymphocytes. *Cell. Immunol.* **7,** 459.

GYÖNGYÖSSY, M.I.C. & PLAYFAIR, J.H.L. (1973) Indirect immunofluorescence of mouse thymus-derived cells using heterologous anti-brain serum. *Cell. Immunol.* **7,** 118.

GYÖNGYÖSSY, M.I.C. & PLAYFAIR, J.H.L. (1974) Rosette formation by mouse lymphocytes. I. Demonstration by indirect immunofluorescence of T cells binding sheep erythrocytes. *Clin. exp. Immunol.* **18,** 169.

HENSON, P.M. (1969) The adherence of leukocytes and platelets induced by fixed IgG antibody or complement. *Immunology*, **16,** 107.

HUBER, H. & DOUGLAS, S.D. (1970) Receptor sites on human monocytes for complement: binding of red cells sensitized by cold autoantibodies. *Fed. Proc.* **29,** 631.

HUBER, H., POLLEY, M.J., LINSCOTT, W.D., FUDENBERG, H.H. & MULLER-EBERHARD, H.J. (1968) Human monocytes; distinct receptor sites for the third component of complement and for immunoglobulin. *Science*, **162,** 1281.

JONDAL, M., HOLM, G. & WIGZELL, H. (1972) Surface marker on human T and B lymphocytes. *J. exp. Med.* **136,** 207.

KOMURO, K. & BOYSE, E.A. (1973) Induction of T lymphocytes from precursor cells *in vitro* by a product of the thymus. *J. exp. Med.* **138,** 479.

LAY, W.H. & NUSSENZWEIG, V. (1968) Receptors for complement on leukocytes. *J. exp. Med.* **128,** 991.

LAY, W.H. & NUSSENZWEIG, V. (1969) Ca dependent binding of antigen–19 S antibody complexes to macrophages. *J. Immunol.* **102,** 1172.

LOOR, F. & KINDRED B. (1973) Differentiation of T cell precursor in nude mice demonstrated by immunofluorescence of T cell membrane marker. *J. exp. Med.* **138,** 1044.

MICHLMAYS, G. & HUBER, H. (1970) Receptor sites for complement on certain human peripheral B lymphocytes. *J. Immunol.* **105,** 601.

NUSSENZWEIG, V., BIANCO, C., DUKOR, P. & EDEN, A. (1971) *Progress in Immunology* (ed. by B. Amos), p. 73. Academic Press, London.

PINCUS, S., BIANCO, C. & NUSSENZWEIG, V. (1972) Increased proportion of CRL in the peripheral blood of patients with CLL. *Blood*, **3,** 303.

PLAYFAIR, J.H.L. (1968) Strain differences in the immune response of mice. I. The neonatal response to sheep red blood cells. *Immunology*, **15,** 35.

ROSS, G.D., RABELLINO, E.M., POLLEY, M.J. & GREY, H.M. (1973a) Combined studies of complement receptor and surface immunoglobulin-bearing cells and sheep erythrocyte rosette-forming cells in normal and leukemic human lymphocytes. *J. clin. Invest.* **52,** 377.

ROSS, G.D., POLLEY, M.J., RABELLINO, E.M. & GREY, H.M. (1973b) Two different complement receptors on human lymphocytes. *J. exp. Med.* **138,** 1973.

SCHLOSSMANN, S.F. & HUDSON, L. (1973) Specific purification of lymphocyte populations on a digestible immunoabsorbant. *J. Immunol.* **110,** 313.

SILVEIRA, N.P.A., MENDES, M.F. & TOLNAI, M. (1972) Tissue localization of two populations of human lymphocytes distinguished by membrane receptors. *J. Immunol.* **108,** 1456.

SOTERIADES-VLACHOS, C., GYÖNGYÖSSY, M.I.C. & PLAYFAIR, J.H.L. (1974) Rosette formation by mouse lymphocytes. III. Receptors for immunoglobulin on normal and activated T cells. *Clin. exp. Immunol.* **18,** 187.

UHR, J.W. (1965) Passive sensitization of lymphocytes and macrophages by antigen–antibody complexes. *Proc. nat. Acad. Sci. (Wash.),* **54,** 1599.

UHR, J.W. & PHILLIPS, Y.M. (1966) *In vitro* sensitization of phagocytes and lymphocytes by antigen–antibody complexes. *Ann. N.Y. Acad. Sci.* **129,** 793.

WORTIS, H.H., NEHLSEN, S. & OWEN, J.J. (1971) Abnormal development of the thymus in 'nude' mice. *J. exp. Med.* **134,** 681.