Immunology 1976 **31** 249

Helper T-cell kinetics and investigation of antigen receptor expression on early and memory T-helper cells

R. MOHR* & U. KRAWINKEL *Institute for Genetics, University of Cologne, Federal Republic of Germany*

Received 17 *November* 1975; *accepted for publication* 9 *February* 1975

Summary. The subcutaneous application of soluble antigen resulted in generation of helper T lymphocytes in the draining lymph nodes, tested 4 days after priming in the adoptive transfer system. Four days later a specific helper T-cell population was detected in the spleen. B-cell activity could be demonstrated 4–5 days after helper T-cell activity in both organs.

We investigated the early helper T-cell population and memory T cells with respect to differences in the expression of antigen receptors. No such differences could be detected, either by anti-immunoglobulin-coated Degalan columns or by an antigen-specific rosetting method.

INTRODUCTION

Following administration of a particulate antigen there is rapid generation of helper T lymphocytes which participate in the ensuing primary immune response. Accompanying the appearance of primary T helper cells is the generation of a long-term reactive T memory cell population. It is not known whether the T memory cell develops from the primary T helper cell or whether these cells are the products of two independent differentiation pathways.

* Present address and correspondence: D-5 Köln 41, Josef Stelzmannstr., Medizinische Universitätsklinik, Germany.

Preliminary experiments indicated that subcutaneous injection of BSA resulted in an early generation of helper T lymphocytes in the draining lymph node. Several days later a specific helper T-lymphocyte population was detected in the spleen. Thus we considered it possible that these two temporally and spatially separated T-cell populations represented respectively the primary helper T lymphocyte and the long lived T memory cell. Using an adoptive transfer system we have studied the kinetics of generation of these two cell populations along with the development of memory in the B-cell compartment.

We have investigated whether the helper T cells produced in the draining lymph node early in the response differ with respect to antigen receptors from those arising later in the spleen.

MATERIALS AND METHODS

Mice

Six to 10 week-old CBA mice, male and female were obtained from the Jackson Laboratory, Bar Harbor, Maine, U.S.A.

Antigens

Bovine Serum albumin (BSA) (Behringwerke, Marburg, FRG) and ovalbumin (OA) (Miles Laboratories) were used as antigens. NIP_{10}–OA and NIP_{14}–BSA were prepared according to Brownstone, Mitchison and Rivers (1966).

Immunization
Mice were primed by either: (a) a single i.p. injection of 800 μg per host alum-precipitated BSA or 100 μg per host alum-precipitated NIP_{10}–OA together with 2×10^9 inactivated *Bordetella pertussis* organisms (Behringwerke); or (b) by a single injection of 50 or 500 μg BSA per host in Freund's complete adjuvant (Difco) into the hind footpads. Popliteal and inguinal lymph node cells as well as spleen cells were used 2–32 days after immunization for adoptive transfer.

Cell transfer (Mitchison, 1971)
2×10^7 lymph node cells as well as spleen cells from carrier-primed mice were transfered i.p. into hosts together with 2×10^7 hapten-primed spleen cells. There were six mice per group. The hosts received 500 rad X-ray irradiation one day before transfer. Antigen (NIP_{10}–OA or NIP_{14}–BSA each 10 μg per host i.p.) was given 12 h after transfer and the mice were bled 10 days later.

Antibody analysis
Antibody estimations were all done by the modified Farr assay (Mitchison, 1971) using ^{125}I-labelled antigens. Plastic tube binding assay was performed by the method of Askenase and Leonard (1970).

Separation of T lymphocytes on Degalan columns (Wigzell, Sundquist and Yoshida, 1972)
Spleen and lymph node cells were separated on MIG-coated Degalan columns which had been incubated with 1:10 diluted rabbit anti-mouse immunoglobulin (MIg) serum. Details have been described previously (Rajewsky and Mohr, 1974).

Separation of antigen-binding cells (Bach and Dardenne, 1972; Parish, personal communication)
Sheep red blood cells (SRBC) were coupled with BSA as described by Jandl and Simmons, (1957). Antigen coupling to erythrocytes was tested in the passive hemagglutination assay with anti-BSA serum.

Lymph node and spleen cells of BSA-primed mice were incubated with BSA-coupled SRBC for rosette formation according to the method described by McConnell, Munro, Coombs and Gurner (1969).

Rosetting and non-rosetting cells were separated on Isopaque–Ficoll separation fluid as described by Thorsby and Bratlie (1970) under modified conditions. Rosetted cells were layered on a separation fluid of one part of 32·8 per cent Isopaque (Nyergaard, Oslo) and 2·4 parts of 14 per cent Ficoll (Pharmacia, Uppsala, Sweden) in distilled H_2O with 0·1 per cent NaN_3. The separation of rosetting and non-rosetting cells was achieved by centrifugation at 1·200 *g* for 15 min at 20°. Cells at the interphase between separation fluid and cell medium are the non rosetting cells whereas rosetting cells sediment to the bottom of the tube. The interphase cells were tested in the adoptive transfer system.

RESULTS

The kinetics of generation of lymph node and splenic T helper cells following subcutaneous immunization

Hapten-primed cells were transferred together with carrier primed cells into sublethally irradiated mice which were then challenged with the hapten–carrier complex (Mitchison, 1971). In this situation the humoral anti-hapten response reflects the activity of carrier-primed helper cells.

In order to generate a carrier-primed T helper cell population mice were immunized in the hind footpads with BSA. At intervals over a period of 3–32 days these animals were killed. The draining popliteal lymph nodes or spleens were harvested and cells obtained from these organs were injected into X-irradiated recipients along with NIP–OA-primed spleen cells. The latter population thus contained hapten-primed PFC precursor cells. These animals were then challenged with NIP–BSA and 10 days later the anti-NIP and anti-BSA antibody responses were measured.

As shown in Fig. 1, helper activity could be demonstrated in the lymph node cells 3 days after priming, but could not be demonstrated in the spleen before day 10. In comparing the kinetics of appearance of a helper cell response and a B-cell response in BSA-primed lymph node cells, we observed that the B-cell response lagged 4–5 days behind the first appearance of helper activity. A similar phenomenon could be observed in the spleen cell population. In this case helper activity was detected 10 days after priming whilst the B-cell response did not appear before day 16.

It is known that by 6 weeks a memory population has fully developed (Mitchison, 1971). Under our experimental conditions both the helper and the

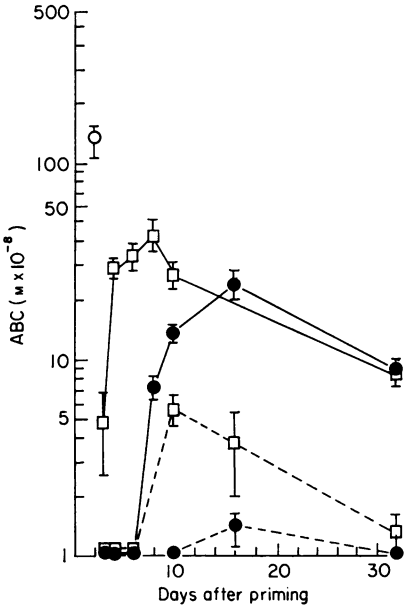

Figure 1. Kinetics of helper activity and precursor activity of lymph node cells and spleen cells after s.c. priming with 50 μg per host BSA. Anti-NIP response after stimulation with homologous hapten–carrier complex (○). Anti-NIP response of primary NIP–OA precursors and primary BSA lymph node helper cells after stimulation with 10 μg per host NIP–BSA (□——□). Anti-BSA response of primary lymph node B cells (●——●). Anti-NIP response of primary precursors and primary BSA spleen cells (from the same donors as the lymph node cells) after stimulation with NIP–BSA (10 μg/host) (□- - -□), and anti-BSA response of the spleen cells (●- - -●).

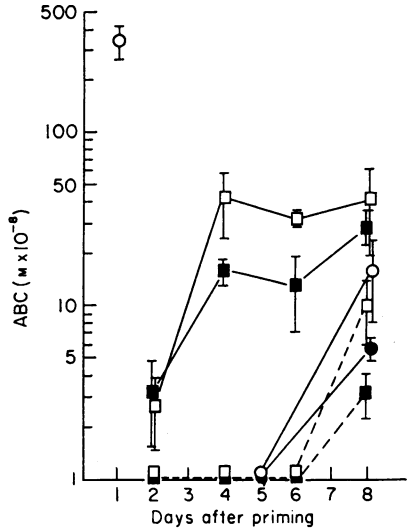

Figure 2. Kinetics of helper activity and precursor activity of lymph node cells and spleen cells after s.c. priming with 50 μg and 500 μg per host BSA. Anti-NIP response after stimulation with homologous hapten–carrier complex (○). Anti-NIP response of primary NIP–OA precursors and primary BSA lymph node helpers cells primed with 50 μg/host (□——□) and 500 μg/host (■——■). Anti-NIP response of primary NIP–OA precursors and primary BSA spleen cells primed with 50 μg/host (○——○) and 500 μg per host (●——●) after stimulation with 10 μg per host NIP–BSA, Anti-BSA response of the lymph node B cells primed with 50 μg/host (□- - -□) and with 500 μg/host (■- - -■) N.

Table 1. Estimation of anti-BSA titres with the Farr assay and the modified plastic tube assay

Group	Helper	Precursor	Antigen			Plastic tube assay*	
	Primary BSA	Primary NIP–OA		NIP ($M \times 10^{-8}$)	ABC–BSA ($M \times 10^{-8}$)	Dilution of Serum pool	Anti-BSA binding (c.p.m.)
1	—	+	—	<0·1	<0·1	n.d.	n.d.
2	—	+	NIP–OA	180·7 (1·2)	<0·1	n.d.	n.d.
3	+	+	—	<0·1	<0·1	n.d.	n.d.
4	+	+	NIP–BSA	11·6 (1·1)	20·2 (1·3)	1:125	23,752±240
5	+	+	NIP–BSA	12·7 (1·2)	<0·1	1:5	546±105

The anti-BSA titres were measured 10 days after adoptive transfer of spleen cells 6 weeks after priming with BSA (group 4) and lymph node cells 4 days after priming (group 5) with BSA. The animals were challenged with 10 μg per host NIP–BSA.
n.d. = Not determined.

* Aliquots of sera from six mice were pooled and serial dilutions were tested for anti-BSA binding in the plastic tube assay. The binding values presented in this table were taken from the linear part of the binding curve and are corrected for background binding (880 c.p.m.).

precursor activity of lymph node and spleen cells decreased 32 days after priming.

In a second experiment mice were primed by injection of 50 or 500 μg BSA per host and the kinetics of development of the response were assayed by transfer up to the 8th day after priming. The kinetics of the response were identical in both cases though the cells from the animals primed with 50 μg of BSA yielded slightly higher anti-NIP and anti-BSA titres. We therefore used the lower dose in the following experiments (see Fig. 2).

Transfer of lymph node cells 4 days after priming did not upon stimulation with NIP–BSA yield an anti-BSA response detectable with the Farr assay. These sera were therefore retitrated using the plastic tube binding assay which is approximately 100 times more sensitive than the Farr assay. As shown in Table 1 no anti-BSA response was detectable even at this level of sensitivity of analysis.

Surface characteristics of T helper cells immediately after priming compared with memory T cells

We examined the possibility that helper cells in an early state of maturation carry cell-bound immunoglobulin receptors. Lymph node cells 4 days after priming were passed over a rabbit anti-mouse IgG-coated Degalan column. The effluent cells were tested for helper activity in the adoptive transfer system. Neither the helper cells present 4 days after priming nor the memory T cells present 6 weeks after priming could be eliminated using this technique (Table 2).

SRBC coated with antigen can be used to form specific rosettes when incubated with primed cells. The rosetting and non-rosetting cells can be separated from each other and the function of each population can then be tested. Whereas unprimed and memory B cells can form specific rosettes, it has been found that the long lived T memory cell does not participate in this reaction (Elliott, Haskil and Axelrad, 1973; Elliott and Haskil, 1975; Krawinkel, unpublished data). On the other hand it is known that some T cells in the primed population are able to form specific rosettes, but that these cells are void of helper activity (Elliott *et al.*, 1973; Elliott and Haskil, 1975).

We therefore investigated the possibility that the helper cells produced early in an immune response would, unlike memory T cells, form antigen-specific rosettes.

Lymph node cells 4 days after priming and spleen cells 6 weeks after priming were rosetted with BSA-coupled SRBC and fractionated into rosetting and non rosetting cells in the Isopaque–Ficoll system. The cell fractions were then tested for helper cell and B-cell activity in the adoptive transfer system. Neither the early helper cells nor the memory helper cells were found in the rosetted cell fraction. In both cases helper activity was totally associated with the non-rosetted cell population. To control both the specificity and the capacity of the rosetting and separation techniques,

Table 2. Spleen cells (groups 4, 5 and $6^{\times 1}$) 6 weeks after priming with BSA and lymph node cells (groups 7, 8 and $9^{\times 2}$) 4 days after priming with BSA were used in the adoptive transfer system together with NIP–OA-primed spleen cells

Group	Helper primary BSA	Column	Precursor primary NIP–OA	Antigen	ABC ($M \times 10^{-8}$)	
					NIP	BSA
1	—	—	+	—	<0·1	<0·1
2	—	—	+	NIP–OA	453·7 (1·2)	<0·1
3	—	—	+	NIP–BSA	<0·1	<0·1
4	$+^{\times 1}$	—	+	—	<0·1	<0·1
5	$+^{\times 1}$	—	+	NIP–BSA	3·39 (1·22)	15·8 (1·32)
6*	$+^{\times 1}$	+	+	NIP–BSA	10·6 (1·35)	2·7 (1·5)
7	$+^{\times 2}$	—	—	—	<0·1	<0·1
8	$+^{\times 2}$	—	+	NIP–BSA	49·8 (1·2)	<0·1
9*	$+^{\times 2}$	+	+	NIP–BSA	54·0 (1·1)	<0·1

* The cells of group 6 and 9 were passaged over a rabbit anti-mouse immunoglobulin-coated Degalan column. Adoptive anti-NIP and anti-BSA response were measured 10 days after transfer and challenge with antigen.

Table 3. Spleen cells (expt 1, groups 4, 5 and 6 and expt 2, group 1) 6 weeks after BSA priming and lymph node cells (expt 1, groups 7 and 8) 4 days after BSA priming were used as helper cells

Expt	Group	Helper primary BSA	Separated non-rosetting cells	Precursor	Antigen	ABC ($M \times 10^{-8}$)	
						NIP	BSA
1	1	—	—	+	—	<0·1	<0·1
	2	—	—	+	NIP–OA	462·0 (1·3)	<0·1
	3	—	—	+	NIP–BSA	<0·1	<0·1
	4	+	—	+	—	<0·1	<0·1
	5	+	—	+	NIP–BSA	86·8 (1·2)	333·4 (1·2)
	6*	—	+	+	NIP–BSA	308·4 (1·2)	3·3 (1·5)
	7	+	—	+	NIP–BSA	332·4 (1·2)	1·9 (1·3)
	8*	—	+	+	NIP–BSA	540·6 (1·5)	<0·1
2	1	+	—	+	NIP–BSA	66·4 (1·1)	64·0 (1·0)
	2†	—	+	+	NIP–BSA	123·3 (1·2)	1·2 (1·1)

* Groups 6 and 8 are helper cells taken from the non-rosetted cell fraction after separation on Isopaque–Ficoll.

† Group 2 in expt 2 represents a mixture of primary NIP–OA precursors and primary BSA helper spleen cells, rosetted together with BSA-coated sheep erythrocytes. After separation by Isopaque–Ficoll separation fluid the fraction of non-rosetted cells was transferred.

cells from animals primed with NIP–OA were mixed with cells from animals primed with BSA, the mixture was rosetted with BSA-coupled SRBC and the rosetted and non-rosetted cell populations separated on the Isopaque–Ficoll system. All NIP-precursor cells could be detected in the non-rosetted cell population which nevertheless was depleted by 99 per cent of the BSA precursors (see Table 3).

DISCUSSION

The kinetics of T and B memory-cell generation following administration of a particulate antigen have been exhaustively analysed *in vivo* (Cunningham and Sercarz, 1971; Kappler and Hoffmann, 1973; Black and Inchley, 1974) and *in vitro* (Kettman and Dutton, 1971). It has been found that helper T memory cells are generated 2–4 days after antigen application and are followed with a lag period of 1–2 days by B memory cells. The ensuing primary PFC response develops approximately at the same time as memory B cells are detectable.

In contrast, little information has been available on the kinetics of memory generation following immunization with a soluble protein antigen, although it is well known that the primary immune response to such an immunogen develops much slower than to a particulate antigen (Nossal, Ada and Austin, 1964).

We have found that memory-cell generation following stimulation with a soluble protein antigen (BSA) occurs almost as rapidly as that for a particulate antigen with respect to helper cell activity. Helper cells appeared first in the draining lymph node 3–4 days after subcutaneous immunization and after a lag period of a further 4 days also in the spleen.

The generation of the B-cell memory in the lymph node cells required 8 days after immunization. Though we did not distinguish in this study between an IgM and IgG response it is known that in the secondary response elicited in the adoptive transfer only an IgG response occurs (Rajewsky and Brenig, 1974).

The time discrepancy between generation of B-cell memory after particulate or soluble antigen stimulus may be explained in several ways. The most trivial explanation would be that in our experiments too few B lymphocytes were transferred with the lymph node cell suspension to give a detectable antibody response. This, however, is rather unlikely, since it has been shown that as few as 3×10^6 primed B cells transferred together with a saturating dose of helper cells can produce a significant quantity of antibody (Rajewsky and Mohr, 1974). In our system we transferred 2×10^7

lymph node cells which contain at least 6×10^6 B cells. Furthermore, we analysed the B-cell immune response using the plastic tube assay, which is about a hundred times more sensitive than the Farr assay. Another possible reason for the delayed appearance of BSA–memory B cells may be that competition for T-cell help could take place between primed hapten-specific memory B cells and newly activated B cells directed against the carrier. However, the observation that in control groups of mice which received only BSA-primed lymph node cells 4 days after priming the kinetics of B-cell response were unaltered, argues against this explanation.

The generation of specifically primed lymphocytes was examined in the spleen, where once again detectable helper cell activity appeared some 4–6 days prior to the emergence of a measurable B-cell response. However, the first appearance of helper cell activity in the spleen lagged 6–8 days behind its first appearance in the lymph nodes. B-cell activity in the spleen was likewise delayed with respect to its appearance in the lymph nodes.

We thus feel that the time lag between generation of B memory cells following stimulation with a particulate or soluble antigen must reflect the intrinsic antigenicity or handling of these two forms of immunogen. Should this be the case, it seems that the T cell is less stringent in its requirements for activation than the B cell. Hence T-cell memory generation on stimulation with a soluble antigen occurs at the same rate as following stimulus with a particulate antigen, whereas B memory cell generation is somewhat delayed. Irrespective of the form of antigenic stimulus it appears that the rate of proliferation and differentiation of helper cells is faster than that of B cells. Indeed it is possible that the generation of the B-memory cell pool is dependent on the prior differentiation of a helper cell population.

Subcutaneous administration of antigen resulted in the generation of B- and T-memory cells first in the draining lymph node and later in the spleen. The splenic memory cells may be derived from the lymph node-primed population by circulation (Sprent, Miller and Mitchell, 1971) or may have arisen *in situ* following the transport of antigen from the stimulated lymph node to the spleen. The fact that the injection of a 10-fold higher dose of antigen did not result in a more rapid generation of memory in the spleen might rule against the latter hypothesis, as indeed would the fact that splenic T- and B-cell memory following subcutaneous injection of the high antigen dose was some what lower than that detected after the lower concentration (Fig. 2). It seems more likely that the splenic memory cells are derived from the recirculating lymphocyte population and that the lower helper and precursor activity within the spleen can be explained simply by a dilution effect.

Collection of these data on the kinetics of helper cell generation were necessary in order to be able to investigate whether the helper T cells produced early in a response differ with respect to the expression of antigen receptors from memory T helper cells.

Some T cells have been shown by autoradiographic techniques to bind antigen specifically, though in these experiments the function of the antigen-binding T cells could not be tested (Ashman and Raff, 1973; Roelants, 1972; Roelants and Ryden, 1974; Roelants, Ryden, Hägg and Loor, 1974). Memory T helper cells neither form antigen-specific rosettes (Elliott *et al.* 1973; Elliott and Haskil, 1975) nor bind to Degalan columns coated with anti-immunoglobulin (Wigzell *et al.*, 1972). Nevertheless, 'hot suicide' experiments have shown that memory T helper cells do indeed bind antigen in a highly specific manner (Basten, Miller, Warner and Pye, 1971; Roelants and Askonas, 1971).

The data we present on the T helper cells produced early in an immune response show that these cells are indistinguishable from memory helper cells in terms of their ability to be retained on Degalan columns coated with antisera to mouse immunoglobulin or their ability to form antigen-specific rosettes. By these criteria we have failed to find any evidence of transient antigen-induced changes in the expression of T helper cell receptors.

ACKNOWLEDGMENTS

We thank Drs S. J. Black, R. S. Jack and K. Rajewsky for helpful discussions and R. Bialdyga and S. Irlenbusch for excellent technical assistance. This work was supported by Deutsche Forschungsgemeinschaft SFB 74.

REFERENCES

Ashman R.F. & Raff M.C. (1973) Direct demonstration of theta-positive antigen-binding cells, with antigen induced movement of thymus dependent cell receptors. *J. exp. Med.* **137**, 69.

ASKENASE P.W. & LEONARD E.J. (1970) Solid phase radioimmunassay of human β1C globulin. *Immunochemistry*, **7**, 29.

BACH J.F., & DARDENNE M. (1972) Antigen recognition by T lymphocytes: thymus and marrow dependence of spontaneous rosette forming cells in the mouse. *Cell. Immunol.* **3**, 1.

BASTEN A., MILLER J.F.A.P., WARNER N.L. & PYE J. (1971) Specific inactivation of thymus-derived (T) and non-thymus-derived (B) lymphocytes by ^{125}I-labelled antigen. *Nature: New Biol.* **231**, 104.

BLACK S.J. & INCHLEY C.J. (1974) Characteristics of immunological memory in mice: I. Separate early generation of cells mediating IgM and IgG memory to sheep erythrocytes. *J. exp. Med.* **140**, 333.

BROWNSTONE A., MITCHISON N.A. & PITT-RIVERS R. (1966) Chemical and serological studies with an iodine—containing synthetic immunological determinant 4-hydroxy-3-iodo-5-nitophenylacetic acid (NIP) and related compounds. *Immunology*, **10**, 481.

CUNNINGHAM A.J. & SERCARZ E.E. (1971) The asynchronous development of immunological memory in helper T and precursor B cell lines. *Europ. J. Immunol.* **1**, 413.

ELLIOT B.E. & HASKILL J.S. (1975) Rosette-forming ability of thymus derived lymphocytes in cell mediated immunity: II. Helper cell activity. *J. exp. Med.* **141**, 600.

ELLIOT B.E., HASKILL J.S. & AXELRAD M.A. (1973) Thymus-derived rosettes are not helper cells. *J. exp. Med.* **138**, 1133.

JANDL J.H. & SIMMONS R.L. (1957) The agglutination and sensitization of red cells by metallic cations: interactions between multivalent metals and red-cell membrane. *Brit. J. Haemat.* **3**, 19.

KAPPLER J.W. & HOFFMAN M. (1973) Regulation of the immune response. III. Kinetic differences between thymus- and bone marrow-derived lymphocytes in the proliferative response to heterologous erythrocytes. *J. exp. Med.* **137**, 1325.

KETTMAN J. & DUTTON R.W. (1971) Radioresistance of the enhancing effect of cells from carrier immunized mice in an *in vitro* primary immune response. *Proc. nat. Acad. Sci. (Wash.)*, **69**, 699.

MCCONNELL I., MUNRO A., COOMBS R.R.M. & GURNER, B.W. (1969) Studies on actively allergized cells: the cyto-dynamics and morphology of rosette forming lymph node cells in mice and inhibition of rosette-formation with antibody to mouse immunoglobulin. *Int. Arch Allergy*, **35**, 209.

MITCHISON N.A. (1971) The carrier effect in the secondary response to hapten protein conjugates. I. Measurement of the effect with transferred cells and objections to the local environment hypothesis. *Europ. J. Immunol.* **1**, 10.

NOSSAL G.J.V., ADA G.L. & AUSTIN C.M. (1964) Antigens in immunity. II. Immunogenic properties of flagellin, polymerized flagellin and flagella in the primary response. *Aust. J. exp. Biol. med. Sci.* **42**, 283.

RAJEWSKY K. & BRENIG C. (1974) Paralysis to serum albumins in T and B lymphocytes in mice. *Europ. J. Immunol.* **4**, 120.

RAJEWSKY K. & MOHR R. (1974) Specificity and heterogeneity of helper T cells in the response to serum albumins in mice. *Europ. J. Immunol.* **4**, 111.

ROELANTS G.E. & ASKONAS B.A. (1971) Cell cooperation in antibody induction: the susceptibility of helper cells to specific lethal radioactive antigen. *Europ. J. Immunol.* **1**, 151.

ROELANTS G.E. (1972) Quantification of antigen specific T and B lymphocytes in mouse spleens. *Nature New Biol.* **236**, 252.

ROELANTS G.E. & RYDEN A. (1974) Dose dependence of antigen binding to B and T lymphocytes. *Nature (Lond.)*, **247**, 104.

ROELANTS G.E., RYDEN A., HÄGG L.B. & LOOR F. (1974) Active synthesis of immunoglobulin receptors for antigen by T lymphocytes. *Nature (Lond.)*, **247**, 106.

SPRENT J., MILLER J.F.A.P. & MITCHELL G.F. (1974) Antigen induced selective recruitment of circulating lymphocytes. *Cell. Immunol.* **2**, 171.

THØRSBY E. & BRATLIE A. (1970) *Histocompatibility Testing*, (ed. by P.F. Terasaki), p. 655. Munksgaard, Copenhavn.

WIGZELL H., SUNDQUIST K.G. & YOSHIDA T.O. (1972) Separation of cells according to surface antigens by the use of antibody-coated columns: fractionation of cells carrying immunoglobulins and blood group antigen. *Scand. J. Immunol.* **1**, 75.