Immunology 1985 **56** 269

Suppression of induced erythrocyte autoantibodies is dependent on Lyt 1 cells

PATRICIA HUTCHINGS,* S. MARSHALL-CLARKE† & ANNE COOKE* **Department of Immunology, Middlesex Hospital Medical School, London, and †Department of Medical Cell Biology, University of Liverpool, Liverpool*

Accepted for publication 14 May 1985

Summary. When normal mice are immunized with rat RBC, autoantibodies to mouse red cells and antigen-specific suppressor cells are generated. Suppressor cell activity is found in T-enriched populations and suppresses only the induction of autoantibody, not ongoing or secondary autoantibody responses. Using antibodies lytic for either B cells or distinct T-cell subpopulations, we are able to show that suppression is dependent on the presence of Lyt 1^+ T cells.

INTRODUCTION

Normal mice immunized with rat RBC develop antibodies against their own red blood cells and also suppressor cells capable of preventing the induction of autoantibodies on transfer to naive recipients (Naysmith & Elson, 1977; Cooke, Hutchings & Playfair, 1978; Gare & Cox, 1978). These suppressor cells, present in the spleens of rat RBC-immunized animals, prevent induction of autoantibodies but appear unable to switch off ongoing autoantibody responses or to prevent secondary induction of autoantibodies.

Suppressor cell activity is found in T-cell enriched populations of primed spleen cells. However, since purification of T cells either by nylon-wool columns and/or by 'panning' on petri-dishes coated with antibody to mouse immunoglobulin does not remove all B cells, some workers have attributed the observed suppression to these residual B-cell populations (Naysmith, Ortega-Pierres & Elson, 1981). Therefore, in the experiments reported here, we have employed monoclonal lytic anti-Lyt 1 and Lyt 2 antibodies to T-cell subsets and a lytic monoclonal reactive with B cells in an attempt to try to resolve the question of identity of the cells mediating the observed suppression.

Abbreviations: BA, *Brucella abortus*; Br. MRBC, bromelein-treated mouse red blood cells; Con A, concanavalin A; DCT, direct Coombs' test; DNP, dinitrophenyl; FCS fetal calf serum; LPS, lipopolysaccharide; PFC, plaque-forming cell; Rat RBC, rat red blood cells; SRBC, sheep red blood cells; TNP, trinitrophenyl.

Correspondence: Dr Patricia Hutchings, Dept. Immunology, Middlesex Hospital Medical School, 40–50 Tottenham Street, London W1P 9PG, U.K.

MATERIALS AND METHODS

Mice

CBA/Ca mice were purchased from the National Institute for Medical Research (Mill Hill, London). Wistar rats were bred at the Middlesex Hospital Medical School.

Antisera

The following antibodies were used: monoclonal anti-Lyt 1.1 and anti-Lyt 2.1 were purchased from

New England Nuclear, Boston, MA (used at 1:400). Cells were incubated at 2×10^7/ml for 30 min at 37°, washed once and then incubated with absorbed guinea-pig complement (1/5) for 40 min at 37°. Monoclonal rat antibody LR-1 was derived following fusion of splenocytes from a Lou rat immunized with the rat myeloma cell line Y3 (Galfre, Milstein & Wright, 1979). LR-1 reacts with 50–60% of murine spleen cells and shows no overlap with cells reactive with anti-Thy 1 monoclonal NIM-R1 (Chayen & Parkhouse, 1982). Spleen cells were treated with ammonium chloride to lyse the erythrocytes and were incubated at $1–2 \times 10^7$ cells/ml with LR-1 ascitic fluid at 1:500 on ice. They were washed once in RPMI and incubated for a further 1 hr at 37° with 1:5 diluted fresh guinea-pig serum as a source of complement. The cells were then washed three times, counted and assesed for viability. The cells remaining after treatment with LR-1 were 1–3% surface immunoglobulin positive and more than 85% Thy 1 positive.

Mitogen assays

Spleen cells were cultured in RPMI-1640 (Flow Labs, Irvine, Ayrshire) supplemented with 5 mM glutamine, 2-mercaptoethanol (5×10^{-5} M), 100 μg/ml) streptomycin, 100 units/ml penicillin G, 5 mM HEPES and 5% heat-inactivated FCS (Flow). Cells were cultured at 10^5 per well in 200 μl with Con A (2 μg/ml) or LPS (*E. coli* 055.B5; Difco, Detroit, MI) at 10 μg/ml. The cells were pulsed with 1 μCi [^{3}H]thymidine (TRK61, Amersham International, Amersham, Bucks) at 68 hr and harvested at 72 hr.

Assay of responses to T-independent (T-1) antigens

Cells, 5×10^5, were cultured in 200 μl RPMI-1640 with supplements as above, and TNP-conjugated *Brucella abortus* (TNP-BA) or DNP-LPS.TNP-BA was a 1/10 dilution of the stock prepared according to the method of Mond *et al.* (1978). DNP-LPS (8×10^{-8} mols DNP/mg LPS) was prepared by activation of LPS with cyanogen bromide followed by subsequent reaction with DNP-lysine (BDH). After 4 days in culture, the cells were assayed for PFC against TNP-conjugated SRBC prepared according to the method of Rittenberg & Pratt (1969).

Assay for responses against bromelein-treated mouse red blood cells (Br.MRBC)

Cells, 2×10^5, were cultured in 200 μl RPMI-1640 with supplements as above, and assayed on Day 3 for PFC against Br.MRBC which were prepared according to the method of Cunningham (1974).

Induction and assay of erythrocyte autoantibodies

Red cell autoantibodies were induced by injecting 2×10^8 washed rat RBC i.p. four times at weekly intervals, as described previously (Cooke *et al.*, 1978). Mice were bled from the retro-orbital sinus and the red cells washed four times in isotonic saline before testing for the presence of autoantibodies by an indirect Coombs' test. A single batch of rabbit anti-mouse immunoglobulin (shown by immunoelectrophoresis to react with both IgM and IgG) was incubated with the washed red cells at a final dilution of 1:80. After 30 min incubation with antiserum at room temperature, agglutination was scored microscopically on a scale ranging from 1(positive, visible only under the microscope) to 4 (massive agglutination involving all the cells). This rapid qualitative assay has been shown to correlate closely with a more quantitative but laborious radioimmunoassay (Hutchings, Naor & Cooke, 1985).

Suppressor cell assay

Mice were given four weekly i.p. injections of 2×10^8 rat RBC and, 8–10 weeks later, their spleens were removed and a single cell suspension prepared. For suppression, 40×10^6 of these cells were injected i.v. into normal syngeneic mice 1 day before the first of four rat RBC injections.

Removal of B cells on nylon-wool columns

Briefly, T cells were enriched by passing spleen cells over a nylon-wool column according to a modification of the method of Julius, Simpson & Herzenberg (1973).

Staining for surface immunoglobulin

Cells, 2×10^6, were incubated with 100 μl fluoresceinated rabbit anti-mouse immunoglobulin for 30 min on ice. After washing, the cells showing surface fluorescence were counted as a percentage of the total.

Statistics

Statistical analysis of the data was performed using a Wilcoxon ranking test (Wilcoxon, 1968).

RESULTS

Effect of treatment of rat RBC-primed spleen cells with anti-Lyt 1 and Lyt 2 antibodies

When rat RBC-primed spleen cells are transferred to normal mice, it can be seen that the induced autoantibody response is suppressed (Fig. 1). The response to non-cross reacting determinants on rat RBC is not suppressed (Table 2). When such rat RBC-primed spleen cells are enriched for T cells by passage through nylon-wool columns and treated with lytic antibody to the Lyt 1 antigen prior to transfer, all suppressor activity is removed [there is no significant difference between these animals and controls at Week 4 (Fig. 1)]. In contrast, treatment of the T-enriched spleen cells with lytic antibody to the Lyt 2 antigen leaves suppression intact (Fig. 1) ($P < 0{\cdot}01$ at Week 4).

Effect of treatment of spleen cells with antibody lytic for splenic B cells (LR-1)

Normal mouse spleen cells were treated with LR-1 as described in the Materials and Methods. Control cells were treated with guinea-pig complement only. After treatment, the remaining spleen cells were assessed for their ability to respond to the mitogens LPS and Con A, and to the T-independent antigens TNP-BA and DNP-LPS. Their ability to respond to bromelein-treated MRBC was also assessed (see Materials and Methods for details of these assays).

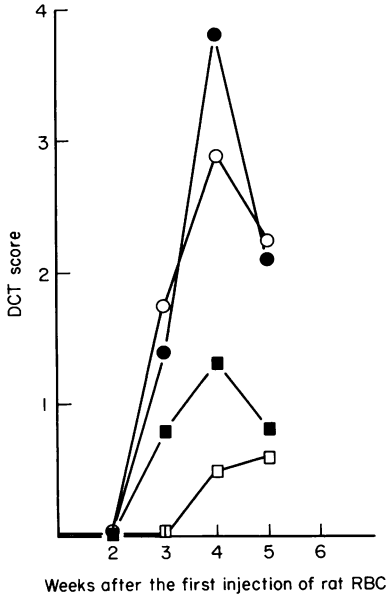

Figure 1. Experiment showing the effect of treatment of rat RBC-primed, T-cell enriched spleen cells with monoclonal anti-Lyt 1 or anti-Lyt 2 antibody and complement on the observed suppression after transfer of these cells to naive recipients which were then given 4 weekly injections of rat RBC (5 mice in each group): (●—●) rat RBC only; (□——□) untreated rat RBC-primed spleen cells; (■——■) anti-Lyt 2-treated rat RBC-primed spleen cells; (○—○) anti-Lyt 1-treated rat RBC-primed spleen cells.

Table 1. Evidence to suggest that LR-1 is specific for splenic B cells

Mitogens/antigen in culture	[^{3}H]thymidine uptake (c.p.m.)	
	Complement only	LR-1 + complement
–	3600 ± 969	969 ± 201
LPS 10 μg/ml	118,917 ± 17,102	4182 ± 1906
Con A 2 μg/ml	228,495 ± 54,726	190,174 ± 23,406
PFC/culture against TNP-SRBC		
–	9 ± 9	0 ± 0
TNP BA 10^{-5}	118 ± 38	1 ± 2
TNP BA 10^{-4}	307 ± 44	7 ± 2
TNP BA 10^{-3}	303 ± 103	± 3·5
DNP-LPS 2 ng/ml	141 ± 38	2 ± 4
DNP-LPS 20 ng/ml	181 ± 48	10·5 ± 5
DNP-LPS 200 ng/ml	368 ± 80	21 ± 20
PFC against Br.MRBC		
–	6·6 ± 11·5	0 ± 0
LPS 20 μg/ml	1266 ± 297	40 ± 40

As can be seen from Table 1, these cells were unresponsive to LPS but their response to Con A was unaffected. They were also unable to respond to TNP-BA or DNP-LPS, and their response to bromelein-treated mouse RBC(Br.MRBC) was virtually abolished.

Effect of treatment of rat RBC-primed spleen cells with LR-1

Rat RBC-primed spleen cells were depleted of B cells by passage through a nylon-wool column. Following treatment of such T-enriched spleen cells with LR-1, significant suppression of the induced erythrocyte autoantibody response was still observed (Fig. 2) ($P < 0{\cdot}01$ at Week 3). When rat RBC-primed spleen cells are transferred to normal mice, and these recipients are challenged with rat RBC, the agglutinin titre in the serum of these animals is usually enhanced compared to those observed in a control group not receiving primed spleen cells. B-cell depletion of these primed spleen cells reduces the rat agglutinin titres observed in the recipients (Table 2).

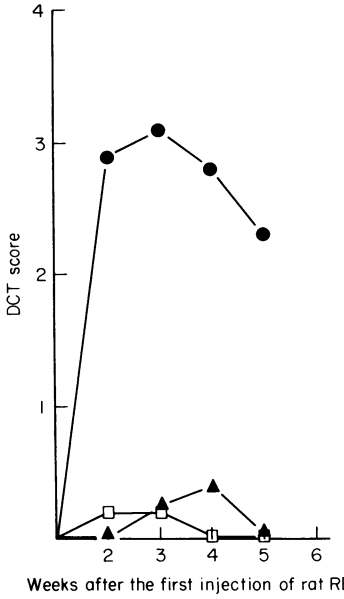

Figure 2. Experiment showing the effect of treatment of rat RBC-primed, T-cell enriched spleen cells with LR-1 (lytic for splenic B cells) and complement on the observed suppression after transfer of these cells to naive recipients which were then given 4 weekly injections of rat RBC (five mice in each group): (●——●) rat RBC only; (□——□) untreated rat RBC-primed spleen cells; (▲——▲) LR-1 treated rat RBC-primed spleen cells.

Table 2. Effect of treatment of rat RBC-primed spleen cells with LR-1 on the agglutination titre in recipients

Treatment of recipients	Anti-rat RBC agglutinating titre in recipients	
	Day 7	Week 5
Rat RBC only	192±70	9216±5154
Untreated rat RBC-primed spleen cells+rat RBC	3157±1533	76,458±26,754
LR-1-treated rat RBC-primed spleen cells+rat RBC	1024±0	28,672±20,561

At both Day 7 and Week 5, the recipients of the LR-1 treated primed cells gave significantly lower responses when compared with the responses of mice given untreated primed cells ($P = < 0{\cdot}01$). There were six mice in each group.

DISCUSSION

Several workers have shown that when spleen cells are transferred from rat RBC-primed mice into normal recipients, the subsequent induction of autoantibody to mouse erythrocytes is depressed. In contrast, the response to determinants on rat RBC which are not shared with mouse RBC is not suppressed and is often enhanced (Naysmith & Elson, 1977; Cooke *et al.*, 1978; Gare & Cox, 1978). It has been suggested that the response to these rat RBC-specific determinants may be suppressed, but such suppression is masked by the presence of primed B or T cells in the transferred primed spleen cell preparations (Cooke *et al.*, 1978). Naysmith *et al.* (1981) have demonstrated the presence of donor allotype in the recipients of primed spleen cells or T-enriched populations prepared by panning on anti-immunoglobulin coated petri-dishes. Our finding that B-cell depletion of the primed spleen cells signficantly reduces the enhanced rat agglutinin response in recipients supports these observations. However, in our hands, such B-cell depletion leaves the suppression of the induced erythrocytes autoantibodies intact.

The data presented in this paper suggest that suppression is dependent on Ly 1 positive cells. It is unlikely that suppression in this system is due to Ly 1 positive B cells, since we find that treatment of peritoneal cells or spleen cells with LR-1 abolishes the response of such treated cells to bromelein-treated mouse RBC. The response to bromelein-treated mouse RBC is known to be the property of Ly 1 positive B cells (Hayakawa *et al.*, 1984). We therefore conclude that the cells mediating suppression are likely to be an Ly 1^+ T cells. The observation that the suppressor function of rat RBC-primed spleen cells is associated with Ly 1^+ cells is similar to the findings of Ly 1^+ T cells suppressing delayed hypersensitivity (Liew & Russell, 1980; Liew *et al.*, 1980; Ramshaw *et al.*, 1977) and Lyt 1^+ suppressor cells determining the outcome of the response of H-2^d mice to *Leishmania donovani* infection (Blackwell & Ulczak, 1984).

Our previous work has suggested that the suppressor cell present in the spleen of animals primed with rat RBC is an inducer of suppression and not a suppressor effector (Hutchings & Cooke, 1981). A similar observation of autoimmunity (experimental allergic encephalomyelitis) being regulated by a suppressor inducer has recently been described in the rat (Pesoa, Hayosh & Swanborg, 1984). In addition, we found that production of autoantibody-secreting B cells is a

prerequisite component of the development of these inducers of suppression (Cooke, Hutchings & Marshall-Clarke, 1980). However, autoantibody production itself does not directly correlate with suppression, since animals making a high autoantibody response do not necessarily develop suppressor function. This is seen in the SJL mouse (Cooke & Hutchings, 1984), in mice treated with a low dose of cyclophosphamide prior to immunization with rat RBC (Hutchings *et al.*, 1985) and in NZB × NZW female mice (Cooke & Hutchings, 1984). It is possible that, in these mice, the defect lies in the development of Ly 1^+ T cells (inducers of suppression).

Some workers have suggested that suppression might be attributable to memory B cells, but we can find no evidence suggesting that SJL mice or cyclophosphamide-treated animals have defective memory B-cell development. We would therefore conclude, that although B cells play a role in the initial development of the suppressor inducer which is an Ly 1^+ T cell, we can find no evidence for a direct role for B cells in the final expression of suppression.

ACKNOWLEDGMENTS

We would like to thank the Medical Research Council and the Wellcome Trust for supporting this research. Anne Cooke is a Wellcome Senior Lecturer.

REFERENCES

BLACKWELL J.M. & ULCZAK O.M. (1984) Immunoregulation of genetically controlled acquired responses to *Leishmania donovani* infection in mice: demonstration and characterization of suppressor T cells in 'noncure' mice. *Infect. Immun.* **44,** 97.

CHAYEN A. & PARKHOUSE R.M.E. (1982) Preparation and properties of a cytotoxic monoclonal rat anti-mouse Thy-1 antibody. *J. immunol. Meth.* **49,** 17.

COOKE A. & HUTCHINGS P. (1984) Defective regulation of erythrocyte autoantibodies in SJL mice. *Immunology*, **51,** 489.

COOKE A., HUTCHINGS P.R. & MARSHALL-CLARKE S. (1980) Lack of autoantigen-specific splenic suppressor cells in mice with an X-linked B-lymphocyte defect. *Immunology*, **41,** 815.

COOKE A., HUTCHINGS P.R. & PLAYFAIR J.H.L. (1978) Suppressor T cells in experimental autoimmune haemolytic anaemia. *Nature (Lond.)*, **273,** 154.

CUNNINGHAM A.J. (1974) Large numbers of cells in normal produce antibody to components of isologous erythrocytes. *Nature (Lond.)*, **252,** 749.

GALFRE G., MILSTEIN C. & WRIGHT B. (1979) Rat x rat hybrid myelomas and a monoclonal anti-Fd portion of mouse IgG. *Nature* **277,** 131.

GARE N.F. & COX K.O. (1978) Erythrocyte autoantibody production by autoantibody induced suppressor cells. *Proc. Aust. Soc. Med. Res.* **11,** 40.

HAYAKAWA R., HARDY R., HONDA M., HERZENBERG L.A., STEINBERG A.D. & HERZENBERG L.A. (1984) Ly-1 B cells: functionally distinct lymphocytes that secrete IgM autoantibodies. *Proc. natn. Acad. Sci. U.S.A.* **81,** 2494.

HUTCHINGS P.R. & COOKE A. (1981) Analysis of the cellular interaction involved in the regulation of induced erythrocyte autoantibodies. *Cell. Immunol.* **63,** 221.

HUTCHINGS P.R., NAOR D. & COOKE A. (1985) Effects of low doses of cyclophosphamide and low doses of irradiation on the regulation of induced erythrocyte autoantibodies in mice. *Immunology*, **54,** 97.

JULIUS M.H., SIMPSON E. & HERZENBERG L.A. (1973) A rapid method for the isolation of funtional thymus derived murine lymphocytes. *Eur. J. Immunol.* **3,** 645.

LIEW F.Y. & RUSSELL S.M. (1980) Delayed-type hypersensitivity to influenza virus: induction of antigen-specific suppressor T cells for delayed-type hypersensitivity to haemagglutinin during infuenza virus infection in mice.

LIEW F.Y., SIA D.Y., PARISH C.R. & MCKENZIE I.F.C. (1980) MHC-coded determinants on antigen specific suppressor factor for delayed-type hypersensitivity and surface phenotypes of cells producing the factor. *Eur. J. Immunol.* **10,** 305.

MOND J.J., SCHER I., MOSIER D.E., BLEASE M. & PAUL W.E. (1978) T independent responses in B cell defective CBA/N mice to *Brucella abortus* and to trinitrophenol (TNP) conjugates of *Brucella abortus*. *Eur. J. Immunol.* **8,** 459.

NAYSMITH J.D. & ELSON C.J. 1977) Autosuppression of an autoantibody response to erythrocytes in mice. *Abstr. Allergologia Immunopathologie*, **5,** 480.

NAYSMITH J.D., ORTEGA-PIERRES M.G. & ELSON C.J. (1981) Rat erythrocyte induced antierythrocyte autoantibody production and control in normal mice. *Immunol. Rev.* **55,** 55.

PESOA S.A., HAYOSH S.N. & SWANBORG R.H. (1984) Regulation of experimental allergic encephalomyelitis. V. Role of the recipient in suppressor cell induction. *J. Neuroimmunol.* **7,** 131.

RAMSHAW I.A., MCKENZIE I.F.C., BRETSCHER P.A. & PARISH C.R. (1977) Discrimination of suppressor T cells of humoral and cell mediated immunity by anti-Ly and anti-Ia sera. *Cell. Immunol.* **31,** 364.

RITTENBERG M.B. & PRATT K.L. (1969) Anti-trinitrophenol (TNP) plaque assay. Primary response of BALB/c mice to soluble and particulate immunogen. *Proc. Soc. exp. Biol. Med.* **132,** 575.

WILCOXON F. (1968) In: *Introduction to Probability and Statistics* (eds H. L. Adler and E. B. Rossler), p. 156. W. H. Freeman and Co., San Francisco.