Clin. exp. Immunol. (1969) **4**, 93–100.

THE EFFECT OF ANTI-LYMPHOCYTIC IgG ON UNSENSITIZED AND SENSITIZED SPLEEN CELLS *IN VIVO* AND *IN VITRO*

K. JAMES, VALERIE S. JAMES AND DIANE M. PULLAR

Department of Surgical Science, The Medical School, Edinburgh

(*Received* 27 *June* 1968)

SUMMARY

Rat spleen cells sensitized to bovine serum albumin and treated *in vitro* with anti-lymphocytic IgG are unable to transfer secondary immunological (humoral) responsiveness to irradiated isogeneic rats. Similar *in vitro* treatment of unsensitized spleen cells has a significant, though less marked effect, upon their ability to confer primary humoral responsiveness to irradiated recipients.

INTRODUCTION

The ability of anti-lymphocytic antibody to suppress both cellular and humoral immune responses in animals treated with this material is well documented (see reviews James, 1967a, 1968a). Cell transfer experiments have also shown that lymphoid cell suspensions prepared from animals treated with anti-lymphocytic antibody, or lymphoid cells which have been treated *in vitro* with this material, exhibit reduced immunological activity when transferred to suitable recipients. For example, such treated cells are less capable of eliciting graft-*versus*-host reactions (Boak, Fox & Wilson, 1967; Monaco *et al.*, 1967; van Bekkum *et al.*, 1967; Brent, Courtenay & Gowland, 1967; Naysmith & James, 1968) rejecting skin allografts (Levey & Medawar, 1967) or producing the normal or sensitized lymphocyte transfer phenomena (Levey & Medawar, 1966, 1967). Furthermore recent studies in mice have shown that anti-lymphocytic antibody treatment of isogeneic bone marrow cells destroys their haemopoietic stem cell function (Demeester, Anderson & Shaffer, 1968).

In addition to modifying cell mediated immune processes, cell transfer experiments have also revealed that the treatment of sensitized lymphoid cells *in vitro* with anti-lymphocytic serum suppresses their ability to transfer humoral responsiveness to irradiated recipients. This effect was originally observed by Harris & Harris (1966) who found that the ability of popliteal lymph node cells from rabbits sensitized to horse spleen ferritin to confer immunological responsiveness to irradiated rabbits was greatly reduced if the cells had been treated *in vitro* with anti-lymphocytic antibody prior to transfer. Similar *in vitro*

Correspondence: Dr Keith James, Department of Surgical Science, The Medical School, Teviot Place, Edinburgh 8.

treatment of spleen cells from adult NZB mice with stong positive antiglobulin reactions also suppressed the development of Coombs positivity in 1-month-old mice receiving these cells (Denman, Denman & Holborow, 1967). Finally preliminary investigations in rats in our own department have indicated that comparable *in vitro* treatment markedly reduced the ability of spleen cells obtained from animals sensitized to bovine serum albumin to transfer secondary humoral responsiveness to irradiated recipients (James, 1968b). The present series of experiments were performed to confirm our previous preliminary observations and to determine the effect of anti-lymphocytic antibody treatment (*in vivo* or *in vitro*) on the humoral antibody producing potential of transferred non-sensitized spleen cells.

MATERIALS AND METHODS

Antisera production

The anti-lymphocytic serum was produced in a horse by the intravenous injection of thoracic duct lymphocytes obtained by cannulation of hooded strain male rats. The horse was injected on days 0, 7, 14 and 28 with between 1·07 and $1{\cdot}3 \times 10^9$ lymphocytes (95% viable) and was bled out on day 38.

Preparation of normal and anti-lymphocytic globulin

Immunoglobulin G preparations were obtained from heat inactivated (56°C for 30 min) normal horse and horse anti-rat lymphocyte sera by the following procedure. The initial step involved the slow addition of 1 volume of 28% (w/v) sodium sulphate to 1 volume of serum. The precipitate was harvested, re-dissolved in physiological saline and re-precipitated as above. This precipitate was then dissolved in and dialysed against 0·02 M-phosphate buffer, pH 6·5, and then further purified by diethylaminoethyl cellulose batch chromatography on a Whatman DE11 cellulose exchanger with an exchange capacity of 1·0 mEq/g. The final product was concentrated by lyophilization, reconstituted in phosphate buffered saline (pH 7·2, 0·06 M-phosphate containing 0·15 M-sodium chloride) and sterilized by filtration through a 0·22 mμ Millipore filter.

Antigen

Crystalline bovine serum albumin supplied by Armour Pharmaceutical Company (list No. 3200) was used throughout these studies. This preparation was reported to contain less than 0·01% contaminating globulin.

Spleen cell donors

The sensitized spleen cells were obtained from 3–6-month-old female rats of an inbred hooded strain which had received intraperitoneal injections of 5 mg of alum precipitated bovine serum albumin, 6 weeks and 2 weeks prior to spleen removal. The normal spleen cells were obtained from 3–6-month-old untreated female rats of the hooded strain. Spleen cell suspensions were obtained by disrupting these organs in cold sterile physiological saline in a ground glass homogenizer followed by filtration through fine mesh stainless steel sieves and washing ($\times 3$) with cold sterile saline.

In vitro treatment of spleen cells

Spleen cell suspensions were incubated for 1 hr at 37°C in sterile physiological saline containing normal horse or anti-lymphocytic IgG (4 mg protein/ml). Each incubation mixture (total volume 20 ml) contained cells from eight spleens, and no extraneous complement was added.

In vivo treatment of spleen cells

Spleen cell donors were injected intraperitoneally with 1 ml of a 2 g/100 ml solution of the appropriate IgG preparation on the 3 consecutive days immediately prior to spleen removal. These cells were incubated in sterile saline alone and all the suspensions were washed three times in this solution prior to transfer.

The viability of the various spleen cell preparations was assessed by their ability to exclude 0·05% Trypan blue and varied between 54 and 88%, the lowest values being observed where the cells had been incubated *in vitro* with anti-lymphocytic IgG.

The preparation and challenge of spleen cell recipients

The recipients of the various suspensions of spleen cells were 3–6-month-old male rats of an inbred hooded strain weighing 220–380 g. All these animals received terramycin in their drinking water (1 mg/100 ml) commencing 2 days before receiving 500 rad of whole body X-irradiation. The irradiation was performed with a 250 kVp Westinghouse therapy unit run at 15 mA. Other radiation factors were 230 kV, constant potential and added filtration of 0·5 mm Cu and 1 mm Al giving a half value layer of 1·2 mm Cu. The final effective dose rate was 61·09 rad/min at a focal skin distance of 75 mm. This dose of radiation had previously been shown to suppress completely the primary response of hooded strain male rats to alum precipitated bovine serum albumin, the antibody assay procedure (see later) revealing that serum samples obtained up to 21 days after antigenic challenge failed to bind low dilutions of test antigen (0·02 μg protein nitrogen/test). Twenty-four hours later the rats were injected intraperitoneally with 150×10^6 viable spleen cells. A further 24 hr after cell transfer the animals were challenged with an intraperitoneal injection of 5 mg of alum precipitated bovine serum albumin. The animals were bled at weekly intervals by cardiac puncture and the circulating antibodies were estimated as follows.

Assay of humoral antibody formation

The antigen binding capacities of the rat sera were determined by the ammonium sulphate procedure of Farr (1958) using ^{131}I-labelled bovine serum albumin. The bovine serum albumin was labelled by the chloramine T technique of Hunter & Greenwood (1962) and more than 96% of the radioactivity was precipitated with 10% trichloroacetic acid. The end point of the antibody titrations was that serum dilution which bound 33% of the antigen (containing 0·2 μg of protein nitrogen) and the antigen binding capacities calculated from this value were expressed as the μg of bovine serum albumin nitrogen bound by 1 ml of undiluted rat serum. All the estimations were performed in duplicate and normal rat serum and standard rat antisera to bovine serum albumins were employed as controls.

The effect of anti-lymphocytic IgG on unsensitized spleen cells was statistically assessed by means of the Student's *t*-test using the Bessell correction for small numbers. The antigen binding capacities of the sera obtained on day 28 from animals receiving normal spleen

cells treated *in vitro* with normal horse or anti-lymphocytic IgG were compared and a value of $P<0{\cdot}1$ was taken as significant.

RESULTS

The effect of anti-lymphocytic IgG on sensitized spleen cells in vivo and in vitro

The results of the present series of experiments show that the treatment of sensitized spleen cells *in vitro* with anti-lymphocytic IgG has a marked effect upon their immunological

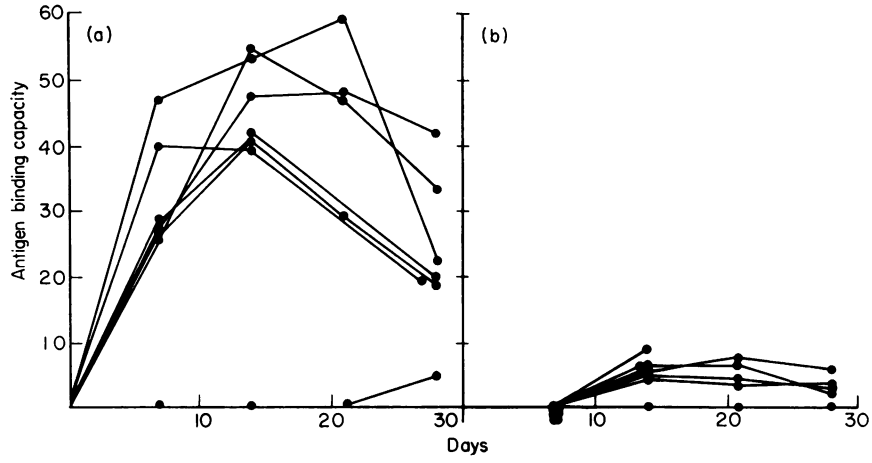

FIG. 1. Cell transfer studies, the effect of anti-lymphocytic IgG on sensitized spleen cells. The figure illustrates the humoral response to alum precipitated bovine serum albumin observed in individual irradiated hooded rats repopulated with isogeneic sensitized spleen cells treated *in vivo* (a) or *in vitro* (b) with anti-lymphocytic IgG. It will be observed that in animals receiving spleen cells treated *in vitro* with anti-lymphocytic IgG the humoral response was greatly reduced. Note also that in many of the early samples the amount of antigen bound was so low that the antigen binding capacity could not be determined with the antigen concentration used. In addition a number of animals died during the investigation period.

activity. Irradiated rats 're-populated' with these treated sensitized cells exhibited a relatively weak humoral response when subsequently challenged with the sensitizing antigen (see Fig. 1b). In contrast a characteristic secondary response was observed following antigenic challenge in irradiated rats which had received sensitized spleen cells from animals treated with a short course of anti-lymphocytic IgG prior to spleen removal (Fig. 1a). The latter response was similar to that previously noted following the transfer of sensitized spleen cells treated *in vivo* or *in vitro* with normal horse IgG (James, 1968b).

The effect of anti-lymphocytic IgG on unsensitized spleen cells in vitro

From Fig. 2 it will be observed that the treatment of unsensitized spleen cells *in vitro* with anti-lymphocytic IgG slightly modifies their subsequent primary humoral response following transfer to irradiated isogeneic recipients. In general there was a delay in the appearance, and a reduction in the quantity of free circulating antibody in animals

receiving these cells (Fig. 2d) when compared to the response observed in animals re-populated with spleen cells treated *in vitro* with normal IgG (Fig. 2b).

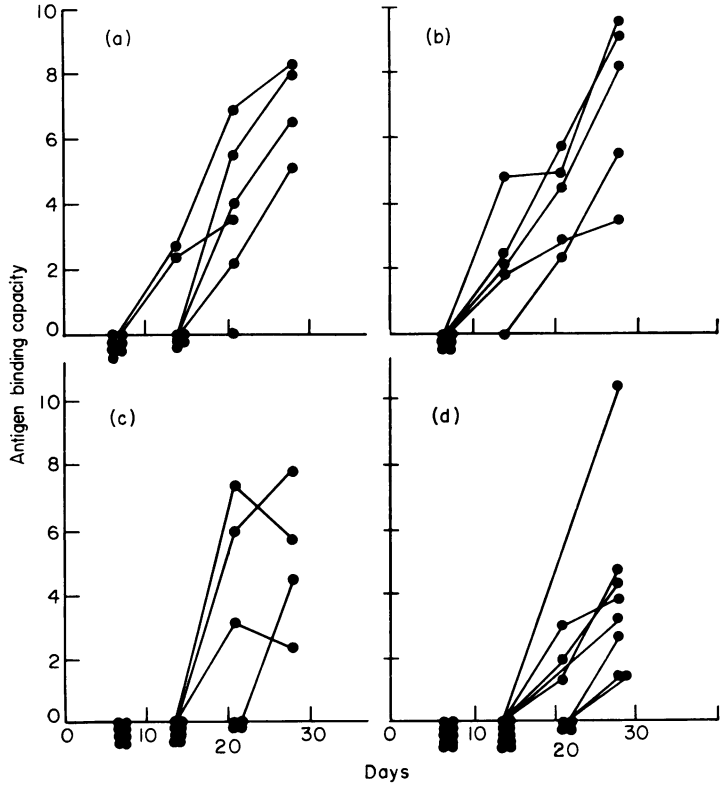

FIG. 2. Cell transfer studies, the effect of normal horse IgG and anti-lymphocytic IgG on unsensitized spleen cells *in vivo* and *in vitro*. The figure illustrates the humoral response to alum precipitated bovine serum albumin in individual irradiated hooded rats re-populated with unsensitized spleen cells treated as follows. (a) Normal IgG *in vivo*, (b) normal IgG *in vitro*, (c) ALS-IgG *in vivo*, and (d) ALS-IgG *in vitro*. It will be observed that the humoral response in (d) was delayed and reduced in comparison to that observed in (b). Note also that in many of the early samples the amount of antigen bound was so low that the antigen binding capacity could not be determined with the antigen concentration used. In addition a number of animals died during the investigation period.

The effect of anti-lymphocytic IgG on sensitized cells in vivo

The *in vivo* treatment of 'unsensitized' spleen cell donors with anti-lymphocytic IgG failed to exert a significant effect upon the immunological activity of the spleen cells following subsequent transfer to X-irradiated recipients. The immune response in animals re-populated with these cells and challenged with alum precipitated bovine serum albumin (Fig. 2c) was similar to that observed in animals receiving spleen cells from donors treated with normal horse IgG (Fig. 2a). In both groups there was a delay in the appearance of free circulating antibodies in a high proportion of the animals.

DISCUSSION

These results clearly confirm the earlier observations from our laboratory and elsewhere that sensitized lymphoid cells (isogeneic or allogeneic) treated *in vitro* with small doses of anti-lymphocytic antibody are unable to restore secondary humoral responsiveness to irradiated rats (James, 1968b) or rabbits (Harris & Harris, 1966). Furthermore, the data suggests that *in vitro* treatment of unsensitized spleen cells may render them less able to confer primary humoral responsiveness. However, in this case the results are less dramatic, the peak mean response being just significantly different from that observed in animals receiving normal horse IgG treated spleen cells.

As previously discussed (James, 1968b) these observations suggest that the *in vitro* treatment of sensitized spleen cells with anti-lymphocytic antibody results in the erasure of immunological memory, presumably as a consequence of memory cell destruction. However, the observed results can be explained on the basis of less selective mechanisms since the recent results of Martin & Miller (1967) have shown that lymphoid cells coated *in vitro* with anti-lymphocytic antibody are 'relatively' ineffective at 're-populating' the lymphoid tissue of irradiated isogeneic recipients. The failure of the anti-lymphocytic antibody treated cells to seed in the lymphoid organs following transfer could have been due to their rapid complement mediated destruction, or as a result of the opsonization and sequestration of intact viable cells. Thus the much reduced immune response in animals receiving anti-lymphocytic IgG treated cells, might have been the direct result of the generalized reduction in effective lymphoid cell repopulation (James, 1968b; Field & Gibbs, 1968).

The ability of sensitized lymphoid cells from animals treated with short courses of anti-lymphocytic IgG, to confer secondary immunological responsiveness upon irradiated recipients, supports previous observations in other rat experiments. It has previously been shown that doses of anti-lymphocytic antibody similar to those used in these experiments were incapable of exerting a marked effect upon the secondary response of rats to alum precipitated bovine serum albumin (James & Jubb, 1967) and also failed to suppress the primary response if administered shortly after the antigen (James, 1967b). All these observations can be explained on the basis of the limited accessibility of anti-lymphocytic IgG to central lymphoid tissues such as the spleen and lymph nodes, its effect being primarily upon the peripheral lymphocytes (Denman *et al.*, 1967; Levey & Medawar, 1967). Thus the antibody is relatively ineffective in inactivating memory cells or preventing plasma cell proliferation in these sites, though it is feasible that much higher doses than used in these experiments could prove effective.

In contrast to the above, studies on the ability of transferred lymphoid tissue to promote skin allograft rejection and graft-*versus*-host reactions, indicate that *in vivo* treatment with small doses of anti-lymphocytic antibody can significantly modify the subsequent cellular response of non-sensitized lymphoid tissue (Boak & Wilson, 1968). It was these observations on cellular systems which prompted us to investigate the *in vivo* and *in vitro* effect of anti-lymphocytic antibody on the humoral antibody producing potential of unsensitized spleen cells. Whilst the *in vitro* anti-lymphocytic IgG treatment had a slight, yet significant effect on the subsequent immunological activity of unsensitized spleen cells, the effects *in vivo* were not significantly different from those of the controls (Fig. 2). However, in all these groups there was a delay in the appearance of free circulating antibodies when

compared to those animals receiving cells treated *in vitro* with normal horse IgG (Fig. 2b) or the response in normal rats (James & Jubb, 1967). This apparent delay in the appearance of free circulating antibodies may have been due to a genuine inactivation of mature antigen reactive lymphoid cells, or their immediate descendants, but was most probably a direct reflection of the small number of cells which seeded, and hence could participate in the immune response. In these circumstances, the low levels of antibody originally produced, could have been bound by the antigen thus stimulating a delayed humoral response.

Although the rate of appearance of free circulating antibody in animals receiving sensitized spleen cells treated *in vitro* with anti-lymphocytic IgG was similar to that observed following primary antigenic stimulation (compare Figs. 1b and 2b), the observed response could be a secondary one effected by a reduced number of active sensitized spleen cells. The decrease in circulating antibody observed 21 days following antigenic challenge is suggestive of a decline in the activity or number of antibody producing cells.

In the present series of experiments the precise interpretation of results has been made difficult by the fact that the spleen cell population is extremely heterogeneous. Hence the percentage of antigen-reactive cells in a standard inoculum might be greatly distorted in anti-lymphocytic IgG treated rats by the proliferation of other cell types, even though the absolute number of lymphocytes remains unchanged. However, studies performed with varying numbers of lymphoid cells obtained from different sources and investigations designed to determine the distribution of anti-lymphocytic antibody and antibody coated cells should further our understanding of the effect of anti-lymphocytic antibody on sensitized and normal lymphoid tissue.

ACKNOWLEDGMENTS

The authors wish to thank Professor M. F. A. Woodruff for his advice and encouragement and Dr N. F. Anderson for irradiating the rats and performing the viable cell counts. They are also indebted to A. Wood and O. Singla for valuable technical assistance, D. Naysmith and B. McBride for helpful suggestions in connection with the manuscript and C. Shepley for preparing the diagrams. Finally one of us (D.N.P.) wishes to acknowledge the generous financial assistance of the Wellcome Foundation.

REFERENCES

BEKKUM, D.W. VAN, LEDNEY, G.D., BALNER, H., PUTTEN, L.M. VAN & VRIES, J. DE (1967) Suppression of secondary disease following foreign bone marrow grafting with anti-lymphocyte serum. *Ciba Study Group on Anti-Lymphocyte Serum*, p. 97. Churchill, London.

BOAK, J.L., FOX, M. & WILSON, R.E. (1967) Activity of lymphoid tissues from anti-lymphocyte serum treated mice. *Lancet*, **i**, 750.

BOAK, J.L. & WILSON, R.E. (1968) Modification of the graft-*versus*-host syndrome by anti-lymphocyte serum treatment of the donor. *Clin. exp. Immunol.* **3**, 795.

BRENT, L., COURTENAY, T. & GOWLAND, G. (1967) Immunological reactivity of lymphoid cells after treatment with anti-lymphocytic serum. *Nature (Lond.)*, **215**, 1461.

DEMEESTER, T.R., ANDERSON, N.D. & SHAFFER, C.F. (1968) The effect of heterologous anti-lymphocyte serum on mouse hemopoietic stem cells. *J. exp. Med.* **127**, 731.

DENMAN, A.M., DENMAN, E.J. & HOLBOROW, E.J. (1967) Suppression of Coombs positive haemolytic anaemia in NZB mice by anti-lymphocytic globulin. *Lancet*, **i**, 1084.

FARR, R.S. (1958) A quantitative immunochemical measure of the primary interaction between I* BSA and antibody. *J. infect. Dis.* **103**, 239.

FIELD, E.O. & GIBBS, J.E. (1968) Cross reaction of anti-lymphocytic serum with haemopoietic stem cells. *Nature (Lond.)*, **217**, 561.

HARRIS, S. & HARRIS, T.N. (1966) Suppression of rabbit lymph node cells by rabbit anti-leucocyte serum demonstrated *in vitro* by antibody plaque tests. *J. Immunol.* **96**, 478.

HUNTER, W.M. & GREENWOOD, F.C. (1962) Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature (Lond.)*, **194**, 495.

JAMES, K. (1967a) Anti-lymphocytic antibody—A review. *Clin. exp. Immunol.* **2**, 615.

JAMES, K. (1967b) Some factors influencing the ability of anti-lymphocytic antibody to suppress humoral antibody formation. *Clin. exp. Immunol.* **2**, 685.

JAMES, K. (1968a) The preparation and properties of anti-lymphocytic sera. *Progr. Surg.* (In press).

JAMES, K. (1968b) *In vivo* and *in vitro* effect of anti-lymphocytic IgG on sensitized spleen cells. *Nature (Lond.)*, **217**, 261.

JAMES, K. & JUBB, V.S. (1967) Effect of anti-rat lymphocyte antibody on humoral antibody formation. *Nature (Lond.)*, **215**, 367.

LEVEY, R.H. & MEDAWAR, P.B. (1966) Some experiments on the action of anti-lymphoid antisera. *Ann. N.Y. Acad. Sci.* **129**, 164.

LEVEY, R.H. & MEDAWAR, P.B. (1967) Further experiments on the action of anti-lymphocytic serum. *Proc. nat. Acad. Sci. (Wash.)*, **58**, 470.

MARTIN, W.J. & MILLER, J.F.A.P. (1967) Site of action of anti-lymphocyte globulin. *Lancet*, **ii**, 1285.

MONACO, A.P., WOOD, M.L., WERF, B.A. VAN DER & RUSSELL, P.S. (1967) Effect of anti-lymphocytic serum in mice, dogs and man. *Ciba Study Group on Anti-Lymphocyte Serum*, p. 111. Churchill, London.

NAYSMITH, J.D. & JAMES, K. (1968) Effect of $F(ab')_2$ from rabbit anti-mouse lymphocyte IgG on the graft versus host reaction in F1 hybrid mice. *Nature (Lond.)*, **217**, 260.