Immunology 1978 **35** 151

Direct blockade of antigen-reactive B lymphocytes by immune complexes. An 'off' signal for precursors of IgM-producing cells provided by the linkage of antigen- and Fc-receptors*

J. OBERBARNSCHEIDT & E. KÖLSCH *Heinrich-Pette-Institut für experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistrasse* 52, 2000 *Hamburg* 20, *Federal Republic of Germany*

Received 6 *October* 1977; *accepted for publication* 31 *October* 1977

Summary. Antigen–antibody complexes efficiently inhibit the induction of antibody formation. Using Mishell-Dutton cultures, it can be demonstrated that neither T cells nor their products are required for this inhibition of IgM PFC formation. The blockade is at the level of B cells and cannot be overcome by LPS or TRF. The data demonstrate that cross-linking of antigen- and Fc-receptors by antigen–antibody complexes is a blocking signal for B cells.

INTRODUCTION

The mechanisms by which lymphocytes perceive 'on' or 'off' signals are still largely unknown despite the fact that they would provide keys to the understanding of immune response regulation. Efficient regulators of immune responses are antigen–antibody complexes (for review, see Uhr & Möller, 1968; Fitch, 1975). Antibodies of the IgG classes (Möller & Wigzell, 1965; Henry & Jerne, 1968; Lang, Nase & Rajewsky, 1969; Dennert, 1971; Kappler, Hoven, Dharmarajan & Hoffmann, 1973), probably IgG_1 (Gordon & Murgita, 1975), together with antigen suppress the induction of a humoral response. This suppression requires an intact Fc part on the antibody molecule (Kappler *et al.*, 1973; Abrahams, Phillips & Miller, 1973; Gordon & Murgita, 1975; Lees & Sinclair, 1975), making it unlikely that covering of antigenic determinants plays an essential role. In fact, it can be assumed that interactions between Fc parts and membrane-bound or secreted Fc receptors are crucial events for blocking.

One model to explain suppression of induction of antibody formation by immune complexes assumes a disturbance of T-B collaboration (Hoffmann, Kappler, Hirst & Oettgen, 1974). A second possibility is blockade of B cells by an Fc-binding suppressive factor from T cells (Gisler & Fridman, 1975). Finally, antigen–antibody complexes could directly block at the B-cell level through interactions with both antigen- and Fc-receptors of antigen-reactive cells (Sinclair & Chan, 1971; Sinclair, Lees & Chan, 1976). The data presented in this paper demonstrate the last model to be correct. Antigen–antibody complexes block directly B cells by a mechanism which cannot be counteracted by triggering or maturation signals (lipopolysaccharide

* Supported by the Stiftung Volkswagenwerk.

Abbreviations used in this paper: B cells, bone marrow-derived cells; Con A, concanavalin A; HA, haemagglutinin; HL, haemolysin; HRBC, horse red blood cells; LPS, lipopolysaccharide; PFC, plaque-forming cells; SRBC, sheep red blood cells; T cells, thymus-derived cells; TNP, trinitrophenyl; TRF, thymus-replacing factor.

Correspondence: Dr J. Oberbarnscheidt, Heinrich-Pette-Institut, Martinistrasse 52, 2000 Hamburg 20, Federal Republic of Germany.

0019–2805/78/0700–0151 $02.00

(LPS) or thymus-replacing factor (TRF)) to these cells.

MATERIALS AND METHODS

Animals

BALB/c/A Bom, DBA/2J Bom mice and nu/nu BALB/c mice were purchased from Gl. Bomholtgard, Ry, Denmark. Young adult animals of both sexes were used.

Antigens

Sheep and horse red blood cells (SRBC, HRBC) were used as antigens. Covalent coupling of red blood cells with trinitrophenyl (TNP) sulphonic acid was according to Kettmann & Dutton (1970).

Antisera

Sera of hyperimmunized mice were used for formation of antigen–antibody complexes. The sera had a haemagglutinin (HA) titre of 2^9–2^{11} (DTT-resistant antibodies). $F(ab')_2$ from rabbit anti-SRBC IgG was prepared according to Weir (1973a; 1973b).

IgG fraction of a rabbit anti-SRBC serum (Behringwerke, Marburg, Germany) was isolated by $(NH_4)_2SO_4$ precipitation (50% saturation) and ion-exchange chromatography on DEAE cellulose.

The combined IgG fractions were lyophilized, dissolved in Walpole's acetate buffer, pH 4·5, digested with pepsin (5 mg/100 mg IgG) for 20 h at 37°. $F(ab')_2$ was isolated by gel filtration on Sephadex G200. The combined fractions were lyophilized, dissolved in and dialysed against BSS. The $F(ab')_2$ preparation had an HA titre of 2^7, but no haemolysin (HL) activity was left.

Anti-Thy 1.2 *treatment of spleen cells*

Anti-Thy 1.2 serum was prepared according to Reiff & Allen (1966). For removal of T cells, 6×10^7 spleen cells were kept in 1 ml of this fluid at a dilution of 1 : 5 in medium for 30 min on ice. Cells were washed and resuspended in the same volume of agarose absorbed guinea-pig complement at a dilution of 1 : 10 in medium, incubated for 30 min at 37°, washed twice, resuspended in medium, and adjusted to the appropriate concentration.

Spleen cell cultures and determination of plaque-forming cells (PFC)

Mishell-Dutton cultures were made according to Mishell & Dutton (1967) and supplemented to 5×10^{-5} with 2-mercaptoethanol (Click, Benck & Alter, 1972). Spleen cells were cultured at a cell density of 1×10^7 nucleated cells/ml. For stimulation, 5×10^6 red blood cells were added. The same number of SRBC coated with antibodies was added in order to measure the influence of antigen–antibody complexes. These complexes were freshly prepared by incubating 1×10^8 SRBC/ml for 1 h with antiserum. The dilution given in the Results section is the actual antiserum dilution used for preparing the complexes.

Supernatants of syngeneic cultures stimulated with concanavalin A (Con A) (Serva, Heidelberg, Germany) were the source for TRF (Wecker, Schimpl, Hünig & Kühn, 1975). TRF together with 20 mg α-methyl-D-mannoside (Fluka, Buchs, Switzerland) was added two days after starting the cultures. Bacterial LPS from *Salmonella typhi* 0901 (DIFCO Laboratories, Detroit, Michigan, USA) was added at the beginning of cultures at a concentration of 25 μg/ml. PFC were assayed using the Jerne technique as modified by Mishell & Dutton (1967) using SRBC or TNP-HRBC (Rittenberg & Pratt, 1969) as target cells. The number of recovered nucleated cells was determined in a Coulter Counter (model D_N; Coulter Electronics, Inc., Hialeah), using Zaponin (Coulter Electronics, Inc.) to lyse red cells. Results will be given as PFC per million recovered nucleated cells.

RESULTS

Time course of PFC appearance under normal or suppressive conditions and specificity studies

The data to be presented in this part are basic for establishing the *in vitro* system of antigen–antibody–mediated suppression and confirm and extend data by other investigators (Kappler *et al.*, 1973; Lang *et al.*, 1969; Abrahams *et al.*, 1973; Hoffmann *et al.*, 1974; Lees & Sinclair, 1975).

The experiment in Fig. 1 demonstrates that antigen–antibody complexes given at the beginning of the culture suppress the appearance of PFC at all subsequent days of cell culturing (Fig. 1). The degree of suppression depends on the concentration of antibodies in the complex (Fig. 2). Only the response to determinants in the antigen–antibody complex (SRBC) or to antigens which can be incorporated into the complex (TNP-SRBC) is

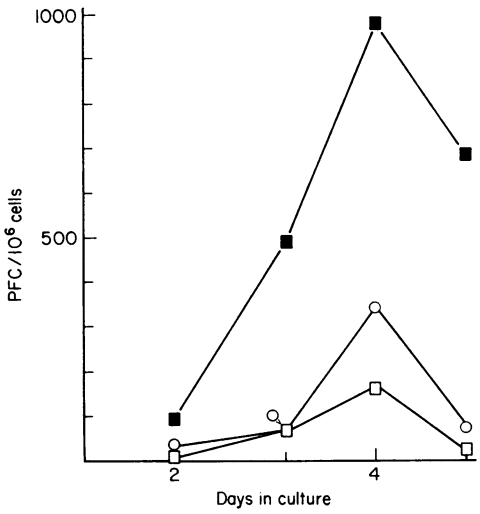

Figure 1. Kinetics of PFC formation in spleen cell cultures under normal conditions and in the presence of antigen–antibody complexes. Ordinate: anti-SRBC PFC/10^6 nucleated spleen cells. Abscissa: days in culture. Spleen cells were cultured in the presence of 5×10^6 SRBC/ml alone (■). Spleen cell cultures contained in addition SRBC anti-SRBC (1 : 5) (□) or SRBC anti-SRBC (1 : 25) (○). HA titre of the undiluted serum was 2^{10}.

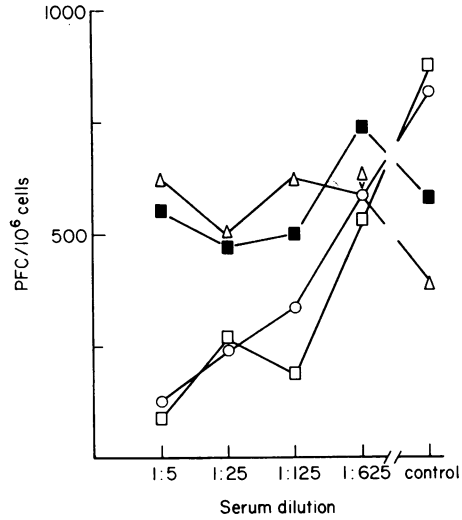

Figure 2. Specificity of suppression by SRBC anti-SRBC complexes. Ordinate: anti-SRBC or HRBC PFC/10^6 nucleated cells. Abscissa: concentration of antibodies in SRBC anti-SRBC complexes (given by the serum dilution used for preparing the complexes). HA titre of undiluted serum was 2^9. Spleen cells were cultured with 5×10^6 SRBC/ml or 5×10^6 HRBC/ml without (control) or with SRBC anti-SRBC complexes. Response to SRBC at day 4 (○) and day 5 (□) and response to HRBC at day 4 (■) and day 5 (△).

reduced (Fig. 2 and Table 1). The response to a third-party antigen (HRBC) in the same culture remains unaffected (Fig. 2). The presence of an intact Fc part on IgG antibodies is a necessary condition (Table 2). The suppression described acts in the initial phase of induction of an IgM response since addition of antigen–antibody complexes 48 h after beginning of the culture is no longer suppressive (Table 3).

Cultures from spleen cells depleted of macrophages by various methods ((Sephadex G10 filtration (Ly & Mishell, 1974), plastic dish adherence (Lee, Chiorawa, Shaw & Diener, 1976), pretreatment of mice with carrageenan (Ishizaka, Otami & Morisawa, 1977)) and supplemented with 2-mercaptoethanol are still inhibitable by immune complexes (data not shown).

Table 1. Suppression of the immune response to TNP-SRBC by SRBC anti-SRBC complexes

Culture conditions*	Anti-SRBC response† Day 4 Exp 1	Anti-SRBC response† Day 4 Exp 2	Anti-SRBC response† Day 5 Exp 1	Anti-SRBC response† Day 5 Exp 2	Anti-TNP response‡ Day 4 Exp 1	Anti-TNP response‡ Day 4 Exp 2	Anti-TNP response‡ Day 5 Exp 1	Anti-TNP response‡ Day 5 Exp 2
(1) TNP-SRBC, SRBC anti-SRBC (1 : 5)	96	18	43	44	26	72	109	33
(2) TNP-SRBC, SRBC anti-SRBC (1 : 25)	129	46	42	24	101	87	44	51
(3) TNP-SRBC, SRBC anti-SRBC (1 : 125)	122	309	88	421	174	260	125	264
(4) TNP-SRBC, nil	418	337	482	626	731	620	665	510
(5) Nil nil	54	16	18	0	70	87	51	33

* Mishell-Dutton culture of DBA/2J Bom ♀ spleen cells with 5×10^6 TNP-SRBC/ml and an equal number of SRBC complexed with antibodies from hyperimmune mouse anti-SRBC antisera (HA titre $\triangleq 2^{11}$). Number in parentheses gives the serum dilution used for preparing the complexes.

† PFC/10^6 nucleated spleen cells developed against SRBC.

‡ PFC/10^6 nucleated spleen cells developed against TNP-HRBC.

Table 2. Lack of suppression of the anti-SRBC response by SRBC anti-SRBC $F(ab')_2$

Culture conditions	Anti-SRBC response* Day 4 Exp 1	Day 4 Exp 2	Day 5 Exp 1	Day 5 Exp 2
(1) SRBC	1011	556	2026	772
(2) SRBC, SRBC anti-SRBC (1 : 50)†	146	38	500	55
(3) SRBC, SRBC anti-SRBC $F(ab')_2$ (1 : 2)‡	587	379	1226	600

* $PFC/10^6$ nucleated spleen cells.
† HA titre 2^{10}.
‡ HA titre 2^6.

Table 3. Influence of the time of addition of SRBC anti-SRBC complexes on the formation of PFC

Culture conditions*	Anti-SRBC response DBA/2 spleen cells† Exp 1	Exp 2	Exp 3	nu/nu BALB/c spleen cells‡
(1) SRBC	1455	595	573	2766
(2) SRBC, SRBC anti-SRBC (1 : 5) 0 h	309	47	48	417
(3) SRBC, SRBC anti-SRBC (1 : 5) 24 h	n.d.§	n.d.	n.d.	650
(4) SRBC, SRBC anti-SRBC (1 : 5) 48 h	3313	680	1118	2122

* SRBC anti-SRBC complexes were omitted (1) or added with the beginning of the culture (2) 24 h (3) or 48 h (4) later. HA titre of the undiluted serum was 2^{11}.
† $PFC/10^6$ nucleated cells measured at day 4.
‡ All cultures of nu/nu BALB/c spleen cells received TRF 48 h after beginning of cultures. $PFC/10^6$ nucleated cells measured at day 5.
§ n.d., not done.

Table 4. Suppression of the immune response to SRBC by SRBC anti-SRBC complexes in cultures of nu/nu BALB/c spleen cells supplemented with TRF

Culture conditions	Anti-SRBC response* Day 4 Exp 1	Day 4 Exp 2	Day 5 Exp 1	Day 5 Exp 2
(1) SRBC, SRBC anti-SRBC (1 : 5)†, TRF	70	96	71	250
(2) SRBC, SRBC anti-SRBC (1 : 25), TRF	85	64	141	276
(3) SRBC, SRBC anti-SRBC (1 : 125), TRF	53	566	188	909
(4) SRBC, nil TRF	563	410	842	1045
(5) Nil nil TRF	n.d.‡	56	n.d.	165

* $PFC/10^6$ nucleated spleen cells developed against SRBC.
† HA titre of the undiluted serum was 2^{11}.
‡ n.d, not done.

Table 5. Suppression of the anti-TNP-SRBC response by SRBC anti-SRBC complexes in cultures of nu/nu BALB/c spleen cells supplemented with TRF or LPS

Culture conditions	Anti-SRBC response* Day 4 Exp 1	Anti-SRBC response* Day 4 Exp 2	Anti-SRBC response* Day 5 Exp 1	Anti-SRBC response* Day 5 Exp 2	Anti-TNP response† Day 4 Exp 1	Anti-TNP response† Day 4 Exp 2	Anti-TNP response† Day 5 Exp 1	Anti-TNP response† Day 5 Exp 2
(1) TNP-SRBC	0	119	48	70	47	94	47	44
(2) TNP-SRBC, LPS	29	278	91	47	191	477	179	107
(3) TNP-SRBC, LPS, SRBC anti-SRBC (1 : 5)‡	0	40	20	5	78	177	48	51
(4) TNP-SRBC, TRF	472	n.d.§	949	n.d.	614	n.d.	1122	n.d.
(5) TNP-SRBC, TRF, SRBC anti-SRBC (1 : 5)	104	n.d.	188	n.d.	300	n.d.	253	n.d.

* PFC/10^6 nucleated spleen cells developed against SRBC.
† PFC/10^6 nucleated spleen cells developed against TNP-HRBC.
‡ HA titre of the undiluted serum was 2^7.
§ n.d, not done.

T-cell independence of suppression by antigen–antibody complexes

Spleen cells from nu/nu BALB/c mice can respond to SRBC or TNP-SRBC in the presence of a TRF-containing supernatant from Con A-activated normal spleen cells. This response can be blocked by anti-SRBC antibodies (Tables 4 and 5). The degree of specific suppression is the same as in normal spleen cell cultures. Specific residual response with SRBC anti-SRBC (1 : 5) is $0{\cdot}06 \pm 0{\cdot}05$ (normal) and $0{\cdot}08 \pm 0{\cdot}04$ (nu/nu) and with SRBC anti-SRBC (1 : 25) $0{\cdot}10 \pm 0{\cdot}08$ (normal) and $0{\cdot}07 \pm 0{\cdot}05$ (nu/nu). These experiments exclude T cells from being directly involved in this type of suppression. However, the existence had to be considered of a suppressive T-cell factor in supernatants of Con A-activated spleen cells, working in combination with antigen–antibody complexes. The fact that also nu/nu BALB/c spleen cells are only suppressed in the initial phase of cell culturing (Table 3) and not 48 h later, at the time when the TRF-containing supernatant was added, makes such an explanation unlikely. However, to test further this remote possibility, the effect of antigen–antibody complexes on nu/nu BALB/c spleen cells in the presence of antigen and LPS was studied. Again it can be shown that antigen–antibody complexes suppress the appearance of PFC (Table 5). In order to exclude the possible argument that anti-Thy-1.2-sensitive immature T cells (Smith & Eaton, 1976) mediate antigen–antibody-induced suppression, the above experiments were repeated after treatment with anti-Thy-1.2 antiserum of both normal and nu/nu BALB/c spleen cells (Table 6). Again immune complexes blocked the triggering of B cells by antigen plus LPS or supernatants from Con A-activated spleen cells (TRF).

Table 6. Suppression of the anti-SRBC response in cultures from anti-Thy-1.2-treated normal or nu/nu BALB/c spleen cells supplemented with TRF or LPS

	Anti-SRBC response* of anti-Thy-1.2-treated BALB/c spleen cells Day 5 Exp 1	Anti-SRBC response* of anti-Thy-1.2-treated BALB/c spleen cells Day 5 Exp 2	Anti-SRBC response* of anti-Thy-1.2-treated BALB/c nu/nu spleen cells Day 4	Anti-SRBC response* of anti-Thy-1.2-treated BALB/c nu/nu spleen cells Day 5
(1) SRBC	153	29	8	132
(2) SRBC, TRF	2195	3944	281	1830
(3) SRBC, TRF, SRBC anti-SRBC (1 : 5)†	263	20	33	200
(4) SRBC, LPS	312	74	112	421
(5) SRBC, LPS, SRBC anti-SRBC (1 : 5)	76	28	46	153

* PFC/10^6 nucleated spleen cells.
† HA titre of the undiluted serum was 2^7.

DISCUSSION

Neither mature nor immature T cells nor their products are required for the *in vitro* blockade of PFC formation by antigen–antibody complexes. There are two requirements for the immune complex being effective in blocking the generation of PFC from antigen-reactive B cells. The hapten determinants impose the selectivity of the blockade, whereas the Fc part of the antibody molecule is the second effector determinant in the complex.

The data shed some light on the function of Fc receptors on B cells. Apparently functional interactions of Fc receptors of B cells with Fc parts of antibody molecules severely restrict the reactivity of B lymphocytes if their antigen receptors are occupied and cross-linked with the Fc receptors. The block induced by the cross-linking of antigen receptor and Fc receptor is dominant over and cannot be overcome by the action of the B-cell mitogen LPS or the helper T-cell replacing factor (TRF) which are likely to exert their function at different stages of B-cell differentiation (Wecker *et al.*, 1975). Our data suggest the block being in an early T-cell-independent phase, possibly already preventing initial proliferation. This contention is further suggested by the finding (Sidman & Unanue, 1976) that proliferative responses of B lymphocytes to LPS are abrogated if these cells have been treated with anti-mouse Ig, a blockade which also requires an intact Fc part in order to be efficient.

Our data stress the importance of antigen–antibody complexes as a way for directly inhibiting B-cell reactivity. This mechanism might be the physiological counterpart to B-cell paralysis by hapten-coupled autologous IgG (Golan & Borel, 1971; Borel, Golan, Kilham & Borel, 1976).

ACKNOWLEDGMENTS

We thank Drs A. Schimpl and E. Wecker for helpful discussions. The expert technical assistance of Ms S. Pröve is acknowledged.

REFERENCES

ABRAHAMS S., PHILLIPS R.S. & MILLER R.A. (1973) Inhibition of the immune response by 7S antibody. Mechanism and site of action. *J. exp. Med.* **137**, 870.

BOREL Y., GOLAN D.T., KILHAM L. & BOREL H. (1976) Carrier determined tolerance with various subclasses of murine myeloma IgG. *J. Immunol.* **116**, 854.

CLICK R.E., BENCK L. & ALTER B. (1972) Enhancement of antibody synthesis *in vitro* by mercaptoethanol. *Cell. Immunol.* **3**, 156.

DENNERT G. (1971) The mechanism of antibody-induced stimulation and inhibition of the immune response. *J. Immunol.* **106**, 951.

FITCH F.W. (1975) Selective suppression of immune responses. Regulation of antibody formation and cell-mediated immunity by antibody. *Prog. Allergy*, **19**, 195.

GISLER R.H. & FRIDMAN W.H. (1975) Suppression of *in vitro* antibody synthesis by immunoglobulin-binding factor. *J. exp. Med.* **142**, 507.

GOLAN D.T. & BOREL Y. (1971) Nonantigenicity and immunologic tolerance: the role of the carrier in the induction of tolerance to the hapten. *J. exp. Med.* **134**, 1046.

GORDON J. & MURGITA R.A. (1975) Suppression and augmentation of the primary *in vitro* immune response by different classes of antibodies. *Cell. Immunol.* **15**, 392.

HENRY C. & JERNE N.K. (1968) Competition of 19S and 7S antigen receptors in the regulation of the primary immune response. *J. exp. Med.* **128**, 133.

HOFFMANN M.K., KAPPLER J.W., HIRST J.A. & OETTGEN H.F. (1974) Regulation of the immune response. V. Antibody-mediated inhibition of T and B cell cooperation in the *in vitro* response to red cell antigens. *Eur. J. Immunol.* **4**, 282.

ISHIZAKA S., OTAMI S. & MORISAWA S. (1977) Effects of carrageenan on immune responses. I. Studies on the macrophage dependency of various antigens after treatment with carrageenan. *J. Immunol.* **118**, 1213.

KAPPLER J.W., HOVEN A.V.D., DHARMARAJAN U. & HOFFMANN M. (1973) Regulation of the immune response. IV. Antibody-mediated suppression of the immune response to haptens and heterologous erythrocyte antigens *in vitro*. *J. Immunol.* **111**, 1228.

KETTMANN J. & DUTTON R.W.J. (1970) An *in vitro* primary immune response to 2,4,6-trinitrophenyl substituted erythrocytes: response against carrier and hapten. *J. Immunol.* **104**, 1558.

LANG W., NASE S. & RAJEWSKY K. (1969) Inhibition of the immune response *in vitro* to sheep red blood cells by passive antibody. *Nature* (Lond.), **223**, 949.

LEE K-C., CHIORAWA C., SHAW A. & DIENER E. (1976) Requirement for accessory cells in the antibody response to T cell independent antigens *in vitro*. *Eur. J. Immunol.* **6**, 63.

LEES R.K. & SINCLAIR N.R.ST.C. (1975) Regulation of the immune response. IX. Resistance to antibody mediated immunosuppression induced by the presence of the allogeneic effect. *Cell. Immunol.* **17**, 525.

LY I.A. & MISHELL R.J. (1974) Separation of mouse spleen cells by passage through columns of Sephadex G10. *J. Immunol. Meth.* **5**, 239.

MISHELL R.J. & DUTTON R.W. (1967) Immunization of dissociated spleen cell cultures from normal mice. *J. exp. Med.* **126**, 423.

MÖLLER G. & WIGZELL H. (1965) Antibody synthesis at the cellular level. Antibody-induced suppression of 19S and 7S antibody response. *J. exp. Med.* **121**, 969.

REIFF A.E. & ALLEN J.M. (1966) Mouse thymic iso-antigens. *Nature* (Lond.), **209**, 521.

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

SIDMAN C.L. & UNANUE E.R. (1976) Control of B-lymphocyte function. I. Inactivation of mitogenesis by interactions with surface immunoglobulin and Fc-receptor molecules. *J. exp. Med.* **144**, 882.

SINCLAIR N.R.StC. & CHAN P.L. (1971) Regulation of the immune response. IV. The role of the Fc-fragment in feedback inhibition by antibody. *Adv. exp. Med. Biol.* **12**, 609.

SINCLAIR N.R.StC., LEES R.K. & CHAN P.L. (1976) Interference with antibody-feedback by irradiation, thymus cells, the allogeneic effect and serum factors. In: *Immune reactivity of lymphocytes: Development, expression and control.* (ed. by M. Feldmann and A. Globerson). Plenum Press, New York, pp. 623.

SMITH J.B. & EATON G.J. (1976) Suppressor cells in spleens from 'nude' mice: their effect on the mitogenic response of B-lymphocytes. *J. Immunol.* **117**, 319.

UHR J.W. & MÖLLER G. (1968) Regulatory effect of antibody on the immune response. *Adv. Immunol.* **8**, 81.

WECKER E., SCHIMPL A., HÜNIG TH. & KÜHN L. (1975) A T-cell-produced mediator substance active in the humoral immune response. *Ann. N.Y. Acad. Sci.* **249**, 258.

WEIR D.M. (1973a) Ion exchange chromatography and gel filtration. In: *Handbook of Experimental Immunology.* 2nd edn. Vol. 1, Ch. 7.8–7.9.

WEIR D.M. (1973b) Cleavage of rabbit and human IgG. In: *Handbook of Experimental Immunology.* 2nd edn, Vol. 1, Ch. 10.16.