EFFECTS OF Fc FRAGMENT ON SURFACE Ig OF MOUSE B CELLS

BY

KARL STEPHAN KRUEGER

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

DEDICATION

To Mutti, Papi, Christiane, Evelyn, Ralph and Birgit

ACKNOWLEDGEMENTS

I am grateful to Dr. Frixos Paraskevas for his continued guidance and valuable advice during the course of this project. I also appreciate the helpful suggestions of Dr. S.-T. Lee, as well as the technical assistance of Pat Sheppard, Peter Ng and Justine DeVries.

ABSTRACT

The role of the Fc receptor on lymphocytes has been variously described as either stimulatory or inhibitory, depending on the system used in measuring the effect of the Fc end of immunoglobulin on its target cell through the FcR. In these experiments, the Fc fragments obtained by papain digestion of either human or rabbit 7S immunoglobulin were allowed to interact with mouse whole spleen cells. The parameter of the spleen cells measured was the presence and distribution of surface immunoglobulin, a feature restricted primarily to B cells. Two methods of detection were used: RICA and FITC-labeled antibody to mouse immunoglobulin. Binding of the Fc fragments was measured using the Fc rosette inhibition assay.

The incubation of rabbit Fc fragments with whole spleen cells for 1/2 hour at 37°C and for 16 - 19 hours at 4°C induced capping and loss of surface immunoglobulin. This indicated that an association, either physical or biochemical, was formed between the FcR and the surface Ig. The human Fc fragments, on the other hand, did not affect the surface immunoglobulin on the B cells, suggesting that only the rabbit Fc, due to its crystalline nature, could bring about sufficient cross-linking of FcR molecules, to affect the surface immunoglobulin.

Since other workers have shown a difference between rabbit and human Fc in terms of their mitogenicity, it was suggested that such a difference could be due to the inhibitory nature of rabbit Fc - FcR interaction. The precise nature of the inhibitory signal was not determined, but an in vivo corollary would be the suppression of specific B cells by antigen-antibody complexes in the presence of antigen excess. These results suggest a major role of the FcR in the immune regulation initiated at the B cell level, after the induction of an antibody response. Such self-regulation would be important in the case of an immune response

which does not lead to the induction of specific T suppressor cells. A lack of cell-mediated suppression would require another form of inhibition, presumably restricted to the B cell and mediated by its surface molecules.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

TABLE OF ABBREVIATIONS

α	= anti
αMIg	= antimouse immunoglobulin
$\alpha\theta$	= antibody to the "theta" surface alloantigen of T cells
B cell	= bursal equivalent - or bone marrow-derived-lymphocyte
BSA	= bovine serum albumin
cAMP	= adenosine 3',5' - cyclic monophosphate
Cγ3	= C3 domain of the heavy chain of IgG
cGMP	= guanosine 3',5' - cyclic monophosphate
CM	= carboxymethyl
DEAE	= diethylaminoethyl
DNA	= deoxyribonucleic acid
EA	= erythrocyte-antibody
expt.	= experiment
Fab	= monovalent antigen-binding fragment of Ig
$F(ab')_2$	= divalent fragment of immunoglobulin lacking the Fc end
Fc	= fragment of immunoglobulin molecule responsible for complement fixation, cytophilic properties and cutaneous attachment
FcR	= Fc receptor
fig.	= figure
FITC	= fluorescein isothiocyanate
g	= force of gravity
γ-G	= IgG
γ-globulin	= IgG
G-100	= type of Sephadex gel (Pharmacia)
H-2	histocompatibility gene locus specifying transplantation antigens
Ia	= I region-determined murine cell surface molecules

Ig	=	immunoglobulin
IgD, IgG, IgM	=	classes of immunoglobulin
IgG1, IgG2b	=	subclasses of IgG
Ig+	=	immunoglobulin-positive
I region	=	immune response region of the major histocompatibility complex of the mouse
LPC-1	=	myeloma cell line of mouse producing IgG2a
LPS	=	lipopolysaccharide
MEM	=	minimal essential medium
mg	=	milligram
MØ	=	macrophage
no.	=	number
O.D.	=	optical density at 280 nm
PBA	=	polyclonal B cell activator
PBS	=	phosphate-buffered saline
RFC	=	rosette-forming cell
RICA	=	reverse immunocytoadherence
S	=	Svedberg unit corresponding to a sedimentation constant of 1×10^{-13} sec./unit of force
7S-γ1, 7S-γ2a	=	IgG1 and IgG2a respectively (subclasses of IgG)
SRBC	=	sheep red blood cells
T cell	=	thymocyte
Thy-1	=	"theta" alloantigen expressed on the T cell surface
TNP	=	trinitrophenol
21-A	=	myeloma of cell line of mouse producing IgG1
UV	=	ultraviolet

INTRODUCTION AND LITERATURE REVIEW

Receptor molecules on lymphocyte and macrophage surfaces have been variously implicated in the mediation of both stimulatory and suppressive signals to B lymphocytes (Schreiner and Unanue, 1976; Dickler, 1976). In this context, Berman and Weigle (1977) have indicated that human, but not rabbit, Fc fragment has a stimulatory effect on the B lymphocyte of the mouse. Such stimulation may be a function of the capacity of these molecules to cross-link the receptors. This study was therefore initiated to determine the effect of human and rabbit Fc fragments on the presence and distribution of surface Ig on mouse B lymphocytes, properties of surface Ig which have been shown to depend on cross-linking of surface molecules (Schreiner and Unanue, 1976).

The property of "certain" lymphocytes to bind immunoglobulin complexed with antigen, without the aid of serum complement, was first observed by LoBuglio et al (1967). The first studies characterizing the binding of immunoglobulin to murine B lymphocytes followed (Basten et al, 1972 a,b,c; Cline et al, 1972; Paraskevas et al, 1972 a,b). Paraskevas et al (1972a) first named the binding molecule on the lymphocyte surface an Fc receptor, because it bound to a site on the Fc region of Ig. Fc receptors have been found on many cells. The cells considered in this paper are lymphocytes (Uhr and Phillips, 1966), which include both B cells (Basten et al, 1972b; Paraskevas et al, 1972a) and "activated" T cells (Yoshida and Andersson, 1972), and macrophages (Howard and Benacerraf, 1966).

The Fc receptor is a non-antigenic marker, according to Golub (1977). It reacts with the Fc portion of the antigen-antibody complex or aggregated immunoglobulin. The receptor can be demonstrated by showing that a complex of erythrocyte and antibody to the erythrocyte (EA) will form rosettes or that labeled immunoglobulin will bind to lymphocytes. In both cases, the binding occurs via the Fc portion of immunoglobulin (Golub, 1977).

The possible biological significance of Fc receptors on mammalian lymphocytes has been reviewed by Kerbel and Davies (1974) and Dickler (1976).

The first to suggest a regulatory role for the Fc receptor (FcR) were Sinclair and Chan (1971). They proposed a tripartite model of antigen, antibody and antigen-sensitive cell. Their model stated that binding of antibody to an antigenic determinant could inhibit or inactivate an antigen-sensitive cell which bound to a neighbouring antigenic determinant on the same molecule or particle of antigen. This inactivation would be mediated through a negative signal transmitted through the Fc portion of the attached antibody.

The Fc receptor of the B cell has been shown to mediate regulation of the humoral response when bound by the Fc portion of specific antibodies (Stockinger and Lemmel, 1978). Also, an antibody irrelevant to the antigen specificity of a particular B cell, linked to the specific $F(ab')_2$ fragment, can mediate the same inhibition through its Fc portion. The inhibitory capacity of an antibody is thought to arise from the cross-linking of surface Ig and Fc receptors by antigen-antibody complexes.

Chan and Sinclair (1971) showed that intact IgG antibody could inhibit the antibody response of B cells. Pepsin-digested $F(ab')_2$ antibody had less ability to suppress the antibody response when it was given one day after the antigen. In the latter case, it was most likely the masking of the antigen which caused suppression of the response.

Havas (1969) has shown that gammaglobulins used as antigens or carriers for haptens were the most effective tolerogens at the time, maybe due to the Fc end of these gammaglobulins helping to inactivate B cells. Köhler et al (1977) found that the Fc portion of an anti-receptor or anti-idiotype antibody was essential for inducing suppression. This would imply cross-linking of the surface immunoglobulin with the FcR, with the anti-receptor antibody acting as a bridge. The participation of T cells was excluded since nude mice were also susceptibile to anti-idiotype suppression. Alternatively, the anti-receptor antibody might bind via its Fc end to macrophages, in which case the macrophage could inactivate the idiotype-bearing B cell. Such anti-idiotype suppression would be particularly important in the

regulation of responses during which no specific T suppressor cells are elicited.

Henry and Jerne (1968) questioned the role of the circulating antibody in immune regulation. They showed that 7S antibody had a suppressive effect, but they proposed that this was due only to the masking of antigen so that it could not bind to the cell. Uhr and Möller (1968) have reviewed results which confirmed the inhibitory capacity of passively administered specific antibodies on the immune response to subsequently administered antigen. More recently, Stockinger et al (1979) have demonstrated inhibition of the immune response to SRBC by administration of spleen cells from a twice-immunized syngeneic donor. The cell responsible for this suppression was found to be a 7S-producing B cell. The T cells and macrophages were not required in the suppressive function of the B cell. This inhibition remained effective for 20 weeks in twice-immunized donors and in recipients after transfer. A similar inhibition was found for passively administered IgG, although this lasted for less than 9 weeks. This suggested that IgG was also mediating the suppressive effect of the 7S-producing B cell.

Among the methods for detection of Fc receptor-bearing cells is the inhibition of RICA (reverse immunocytoadherence). The binding of immune complexes to the cell was found by Paraskevas et al (1972a) to inhibit the detection of surface Ig by RICA. This phenomenon has been investigated here and will be discussed in this paper. The possibility exists that Ig and FcR on the B cell surface cocap, since double labeling of lymphocytes for surface Ig and FcR has shown that a vast majority of Ig-positive cells also bear Fc receptors (Dickler, 1976).

The findings so far on the FcR also include the identification of class and subclass of Ig binding to B lymphocytes. There is little species specificity in such binding, and it is agreed universally that antibodies of the IgG class bind to B lymphocytes. Basten et al (1972c) state that IgG_1 is most strongly bound to the B cell FcR, while Kerbel and Davies (1974) report that IgG2b binding is important for complement-mediated reactions.

Since this paper deals with the interaction between surface Ig and FcR, it

is important to summarize the work on binding to surface Ig by various ligands. First of all, the movement of surface Ig to one pole of the B cell was observed at 37°C following the cross-linking of the Ig with anti-Ig, a process referred to as capping (Taylor et al, 1971). This movement was reported not to induce similar mobilization or cocapping of Fc receptors in human, rat or mouse (Ramasamy and Lawson, 1975). Later experiments, however, found cocapping of FcR when surface Ig was capped (Dickler, 1976). Hence the redistribution of surface Ig affects the distribution of many FcR.

The redistribution of FcR has not been extensively studied. Aggregated Ig or antigen-antibody complexes bound to murine B cells redistribute into large patches and cap at 37°C in a manner analogous to surface Ig (Anderson and Grey, 1974; Abbas and Unanue, 1975; Forni and Pernis, 1975). A relationship does exist between surface Ig and FcR, since capping of surface Ig leads to a redistribution of FcR (Abbas and Unanue, 1975; Forni and Pernis, 1975; Basten et al, 1976). However, in the reverse experiment, capping of FcR does not change the distribution of surface Ig. There is no explanation at present for the association between ligand-bound surface Ig and FcR, but it is possible that membrane changes permit the FcR to cocap with surface Ig.

Since capping of FcR did not change the distribution of surface Ig, the two receptors must normally be independent on the cell surface. Scribner et al (1977) also found that ligand binding to surface Ig induced an alteration in the usual independence of the two receptors, in that both receptors were capped. However, this association was lost on subsequent re-expression and capping of surface Ig. This could relate to the requirement of immune regulation of an activated B cell. The FcR could mediate such regulation only if it was independent of the capping of surface Ig by antigen-binding.

Sidman and Unanue (1975) found that the clearing of surface Ig by anti-Ig antibodies produced a unique suppressive signal in young mice (before 14 days of age). Parker (1975) found that B cells of the mouse proliferate if incubated with anti-Ig antibodies bound, in large amounts, to polyacrylamide beads. This suggested that repeated exposure of the B cell to a dense surface of ligands was

stimulatory. It remains to be seen why these cells did not further differentiate into antibody-secreting cells.

Recently, Hattori et al (1979) have investigated the proliferative response of B cells to intact anti-IgM antibodies, which in their binding mimic the interaction of specific antigen and surface Ig, as well as acting as PBA's. Intact anti-IgM antibodies could lead to B cell proliferation in mice 7 months or older, but not in younger mice. However, the $F(ab')_2$ fragment of anti-IgM was able to stimulate young mice and older mice equally well. It was postulated that the Fc receptors may be involved in the age-dependent response to intact anti-IgM molecules. They also discovered that macrophages were essential for the response to intact anti-IgM in older mice. A suggestion has been made by Hattori et al that there are two subsets of B cells, one susceptible to suppression via the Fc receptor, and the other capable of being stimulated by anti-IgM, with the help of the macrophage. The first subset can be stimulated by the $F(ab')_2$ fragment without the help of macrophages. These are examples of ligand-receptor Ig interactions which provide stimulatory signals to B cells. It should be made clear, though, that only blast formation was studied by Hattori et al, while differentiation itself could be another event (Dutton, 1975).

In contrast, anti-Ig antibodies can inhibit the proliferating and/or differentiating effects of polyclonal B cell stimulatory substances, like LPS. This study has been carried out only in the B cell of the mouse (Elson et al, 1973; Andersson et al, 1974b; Lonai and McDevitt, 1974; Schrader, 1975; Sidman, 1976). Thus far evidence suggests that ligand-receptor Ig interaction programs a brief series of events (including capping and clearing of surface Ig). If the initial response is followed by accessory help, this interaction may stimulate the cell, but without this help, the ligand-surface Ig binding may inactivate the cell.

The state of activation of the B cell can be influenced, therefore, by surface phenomena due to binding of ligand to Ig receptor, or due to binding of

Fc fragment to the Fc receptor. Recently, Berman and Weigle (1977) have shown that the Fc region of immunoglobulin G (IgG), when altered by heat aggregation or when isolated from whole immunoglobulin by papain digestion, can induce strong stimulation of blast formation, DNA synthesis and polyclonal antibody formation. Their system used mouse spleen cells, which were stimulated by human Fc fragment. Subsequently, Berman et al (1979) demonstrated that Fc fragment from mouse IgG could also stimulate DNA synthesis in mouse spleen cells. This was a finding of physiological relevance.

The predominant population of Fc-positive cells in the spleen is the B cell fraction. Although the Fc fragment may act directly on the B cell to produce the effect of DNA synthesis, Morgan and Weigle (1979) have found that the MØ is also essential in this activation process. The binding of Fc fragment from rabbit IgG was first clearly demonstrated by Paraskevas et al (1972a). This has been confirmed in this study. Also, the presence and distribution of γ-globulin on the surface of B cells was affected by the binding of the rabbit Fc fragment to mouse spleen cells. This relationship between surface immunoglobulin and Fc receptor binding was the subject of this study. Two methods used to quantitate the Ig-positive spleen cells were reverse immunocytoadherence (Paraskevas et al, 1971) and fluorescent-labeled antibody to γ-globulin.

The binding of rabbit Fc fragment to mouse spleen cells markedly reduced the percentage of spleen cells with detectable surface γ-globulin. Human Fc fragment, although binding to the Fc receptor, did not affect surface γ-globulin on mouse spleen cells. These observations have been linked to the studies of Berman and Weigle (1977), who found that rabbit Fc fragment does not affect DNA synthesis of mouse spleen cells, whereas human Fc fragment stimulates DNA synthesis. Further lines of investigation are proposed to identify the cause of these differences between human Fc and rabbit Fc.

MATERIALS AND METHODS

Mice: Balb/ c mice, 6-12 weeks old, were obtained from Canadian Breeding, Montreal.

Sheep red blood cells: SRBC were supplied by National Biological Laboratories, Winnipeg.

Antigens: Bovine serum albumin was obtained from Miles Laboratories Inc., Elkhart, Illinois.

Isolation of mouse spleen cells and thymocytes

Spleens or thymuses were removed from 6-12 week old Balb/c mice and teased carefully in a Petri dish in Hanks' solution at $4^{o}C$ (Gibco Canada). The cells were carefully aspirated by Pasteur pipette into a plastic culture tube (Falcon Plastics). They were then washed 2 times at 1500 rpm with Hanks' before the various assays were performed on them.

Viable cell count

Fifty µl of spleen cells in Hanks' were placed in a 2 ml graduated tube, already 3/4 full with Hanks'. Two drops of 1% trypan blue were added and the tube was filled to the mark with Hanks'. The cells were introduced into a hemocytometer chamber, and only the clear cells in the four outer corner squares were counted.

$$\text{Total count} = \#\ \text{cells} \times \text{vol. factor} \times \text{dilution} \times 1000$$
$$= N \times 2.5 \times 40 \times 1000$$
$$= N \times 1 \times 10^{5}\ \text{cells/ml}$$

Red blood cell count

Twenty µl cells were washed in saline until no colouration was present in the supernate and were placed in a 2 ml tube 3/4 full with saline. The tube was then filled to the mark with saline. The suspension was introduced into the hemocytometer and the smaller inner squares were counted.

Total count = # cells in 5 squares X vol. factor X dilution factor X 1000

= N X 50 X 100 X 1000

= N X $5x10^6$

Immunoelectrophoresis

The method was that of Grabar and Burtin (1964). The glass slides (25 x 76 mm) were first coated with 2 ml 0.5% melted agar (Difco Noble) in distilled water and dried at 80°C for 4 hr. In the immunoelectrophoretic tests, 3 ml 2% melted agar in 0.025 M barbital buffer, pH 8.5 were layered on the coated slides. The agar gel was allowed to solidify for a few minutes, and the required wells and troughs were cut. The materials to be tested were placed in the wells and the slides were then placed in the electrophoretic apparatus and run at 14 ma (approx. 70-75 v) for 3 1/2 hr. The slides were then removed from the apparatus, the troughs were filled with the proper antisera and the slides were placed in a moist chamber at room temperature overnight to allow development of the precipitation lines. The slides were washed in 0.9% saline for 24 hr, desalted in distilled water for 8 hr and then dried overnight by placing filter paper on the slides. The dried slides were stained with amido black (1 gm/1000 ml sodium acetate buffer) for 10 min, decolourized in acetic acid (acetic acid: Methanol: water = 150:750 : 750 in vol.) for another 10 min and then air dried.

Ouchterlony

Three ml of 1.6% agar solution in saline was carefully layered onto a glass slide with a 10 ml blow-out pipette, so as to cover the entire slide. The agar was allowed to solidify at room temperature, and with a template, a pattern was cut for six wells surrounding a center well. The circles required to be filled with antigen or antibody were cleared out. The slide was stored in a large Petri dish with a wet cotton ball to prevent drying overnight.

Aggregation of proteins

A solution containing 250 mg of bovine serum albumin (Pentex, Miles Laboratories Inc.) or human IgG (Cohn Fraction II, Miles Laboratories Inc.) or rabbit serum protein in 10 ml of 0.2 M acetate buffer, pH 4.5 was prepared.

Ethyl chloroformate (0.2 ml) was added dropwise with vigorous stirring over a period of 1 min. The mixture was kept stirring for a further 15 min, at room temperature. The stirring was stopped, and the pH adjusted with 1N NaOH between 4.5 and 4.9. The solution was allowed to sit for 1 hr at room temperature, while the pH was checked every 10 min and every 5 min for the last 15 minutes. At the end of 1 hr the pH was brought to 4.5. The aggregate was washed 3X with acetate buffer, pH 4.5, 2X with glycine-HCl, pH 2.5 and 2X with 0.2M phosphate buffer, pH 7.2.

Elution of antibody

A small amount of BSA aggregate prepared as indicated above, was added to 10 ml of anti-BSA serum and the mixture was left stirring overnight at 4°C. The aggregate was centrifuged at 10,000 rpm and the supernate was retained. The aggregate was washed 3X with 0.15 M phosphate-saline, pH 7.2. To dissociate the antibody bound to the aggregate, 10 ml of 0.2 M glycine-HCl, pH 2.5 was added and the mixture was stirred for 1 hr at 4°C. After centrifugation at 10,000 rpm for 1/2 hr, the supernate was collected, since it contained the specific antibody. The antibody solution was immediately neutralized with 0.2 N NaOH and the concentration was determined by optical density at 280 nm. The aggregate was washed 2X with 0.2 M phosphate buffer, pH 7.2 for further use.

Preparation of rabbit Fc fragment

Anti-BSA antibody was prepared from the serum of New Zealand white rabbits immunized at 1 wk intervals for 1 month with 5 mg BSA in Freund's complete adjuvant, and bled from the ear vein 2 wks after the final immunization. The antiserum was first tested by the Ouchterlony Method to ensure a high enough titre. The anti-BSA specific antibody was further eluted with immunoadsorbent, as described above.

The antibody was then digested with the use of 1% papain by weight (mercuri-papain, ICN Pharmaceuticals, Inc.). The papain was dissolved in 0.1 M phosphate buffer, pH 7.0, containing 0.002 M EDTA and 0.01 M cysteine-HCl. EDTA and cysteine-HCl were also added to the antibody solution similarly prepared in 0.1 M phosphate buffer, pH 7.0.

The papain and antibody solutions were incubated before the papain was added to the anti-BSA solution. Digestion was carried at 37°C for 1 hr. The Fc and Fab fragments were separated from the undigested antibody on a G-100 column as described below. The elution profile for papain-digested rabbit IgG is shown in Fig. 1a. The rabbit Fc fragments in the 3.5S peak were isolated by dialysis against cold 0.001 M phosphate, pH 7.0, in which they crystallized. The Fc was then resuspended in phosphate-buffered saline, pH 7.2 after centrifugation at 10,000 rpm for 10 min. The concentration of Fc was measured by diluting a sample into a graduated tube, heating the tube at 45°C for 15 min and reading the optical density at 280 nm in a spectrophotometer.

Preparation of human Fc fragment

Human IgG was obtained from Miles Laboratories Inc., Elkhart, Illinois in the form of Cohn Fraction II and was dissolved with stirring into 0.1 M phosphate, pH 7.0 for papain digestion. The same method of digestion was used as for rabbit IgG, except that the incubation with papain was for 2 hr at 37°C. The elution profile for papain-digested human IgG is shown in Fig. 1b.

The Fc and Fab fragments of papain-digested human IgG were isolated by the method of Franklin (1960), using first a stepwise elution on CM-cellulose. Two peaks were obtained, with 0.01 M phosphate buffer, pH 7.6 as the starting

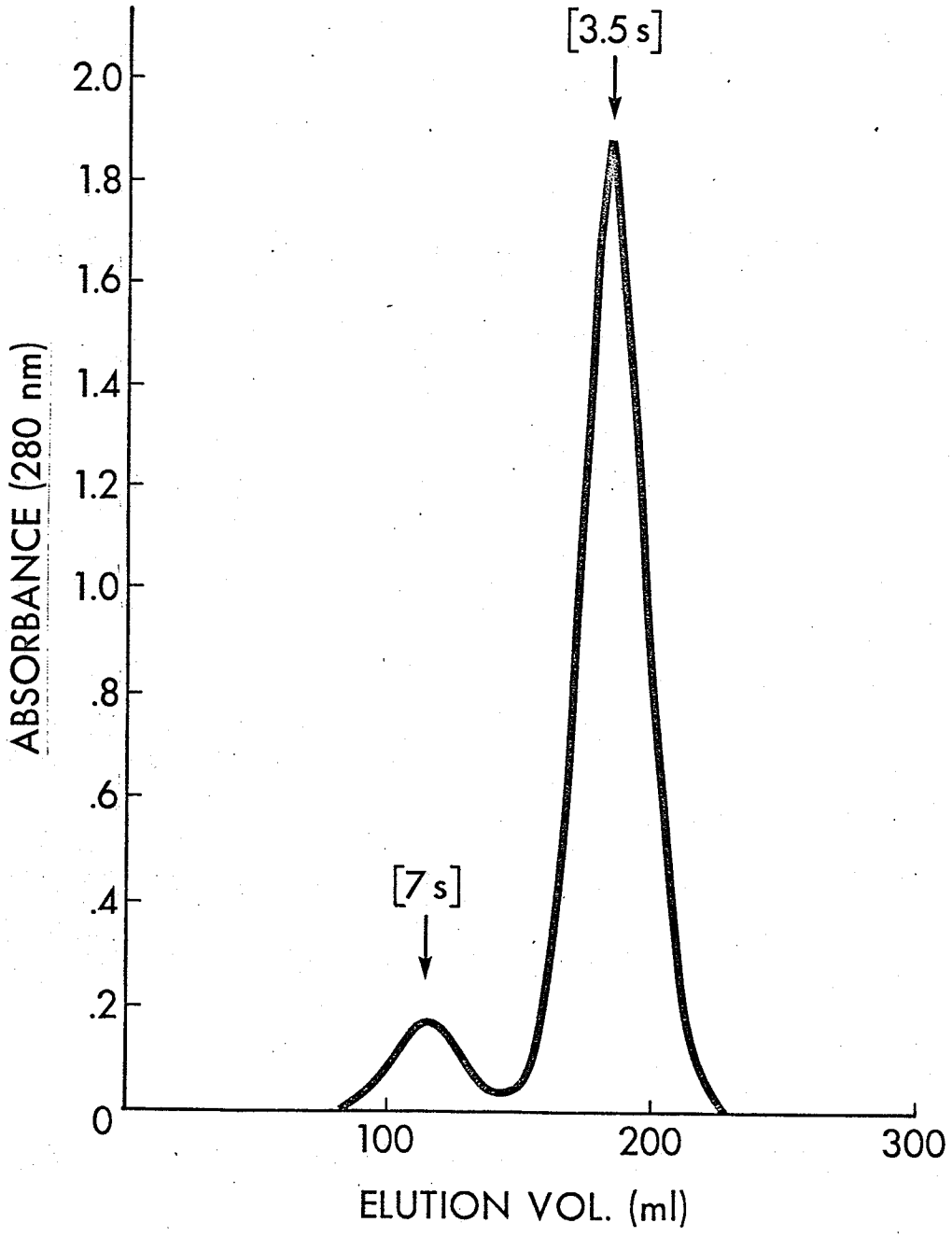

Fig. 1a. Elution profile of papain-digested rabbit anti-BSA on G-100, with 0.15 M borate-saline, pH 8.2 as the eluting buffer.

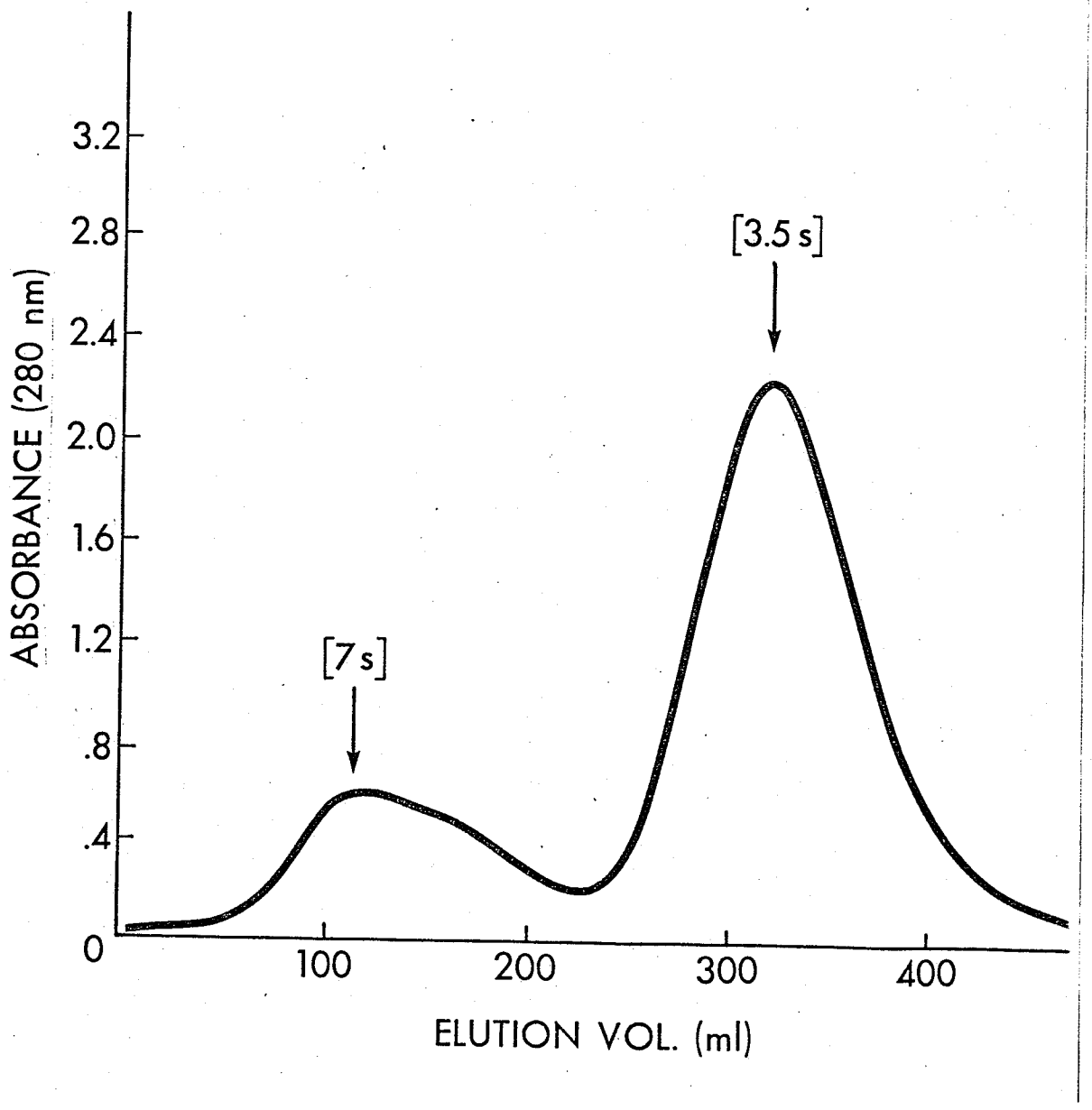

Fig. 1b. Elution profile of papain-digested human IgG on G-100, with 0.15 M borate-saline, pH 8.2 as the eluting buffer.