The Journal of Immunology

RESEARCH ARTICLE | FEBRUARY 01 1980

The Fc gamma receptor on human placental plasma membrane. I. Studies on the binding of homologous and heterologous immunoglobulin G1. FREE

J A van der Meulen; ... et. al

J Immunol (1980) 124 (2): 500–507.
https://doi.org/10.4049/jimmunol.124.2.500

0022-1767/80/1242-0500$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 124, No. 2, February 1980
Printed in U.S.A.

THE Fcγ RECEPTOR ON HUMAN PLACENTAL PLASMA MEMBRANE

I. Studies on the Binding of Homologous and Heterologous Immunoglobulin G[1]

JOHN A. VAN DER MEULEN, TRUDY C. MCNABB, NICOLE HAEFFNER-CAVAILLON,[2] MICHEL KLEIN, AND KEITH J. DORRINGTON

From the Department of Biochemistry, University of Toronto, Toronto, Canada, M5S 1A8

Plasma membrane vesicles prepared from human placental syncitiotrophoblast have been shown to agglutinate IgG-sensitized red blood cells in an Fc specific manner. This agglutination could be inhibited by monomeric IgG and Fc in a dose-dependent fashion, whereas neither IgA nor IgM were inhibitory. This phenomenon formed the basis of an assay that was used to investigate the binding specificity of the Fc receptor. Experiments with human subclass proteins indicated that IgG1 and 3 (and their corresponding Fc fragments) were more effective inhibitors than IgG2 and 4. The ability of IgG from several species to inhibit agglutination was also determined, and the order observed was human IgG1 > rabbit IgG; mouse IgG2a or guinea pig IgG2 > guinea pig IgG1, mouse IgG1, mouse IgG2b, or dog IgG. IgG from sheep, goat, and cow did not inhibit agglutination over the concentration range tested. A role for the hinge region disulfides in maintaining the binding site on IgG is implied by the fact that both IgG or Fc that has been mildly reduced and alkylated and a mutant IgG1 (DOB) that has a hinge region deletion showed a markedly reduced ability to inhibit agglutination compared to the native molecules. A fragment corresponding to the Cγ3 domain of Fc was not able to inhibit agglutination. This observation supports the suggestion that the site on IgG with which the Fc receptor interacts involves both Cγ2 and Cγ3.

A variety of mechanisms are used by mammalian species to confer passive immunity on their young. In humans, maternofetal transfer of antibodies occurs entirely *in utero* and is placenta mediated (1). It has been shown that this transfer process is specific for IgG (e.g., 2, 3) and is mediated by a site located in the Fc region of the antibody molecule (4).

Studies on the specificity of Ig transfer across the rabbit yolk sac first led Brambell (5) to propose a receptor-mediated transport mechanism. According to this hypothesis a receptor for the Fc portion of immunoglobulin is located on the apical membrane of the transporting cell, and a necessary condition for movement of an antibody across the cellular barrier is that it bind to the Fc receptor (FcR)[3]. Many examples of maternofetal transmission of antibody in mammalian species are consistent with such an FcR model (see *Discussion*).

Studies on the FcR of normal human placenta *in vitro* have involved a number of detector systems. Matre and Johnson (6) have looked at the binding of IgG-sensitized red blood cells to cryostat sections. Johnson *et al.* (7, 8) looked at fluorescein-conjugated IgG binding, also using cryostat sections. Both Moskalewski *et al.* (9) and Jenkinson *et al.* (10) have studied binding of IgG-sensitized red blood cells to cell suspensions prepared by trypsinization of placental tissue. In this laboratory (11) we have previously investigated the binding of radiolabeled IgG to placental membrane preparations using centrifugation to separate free ligand from that bound to the membrane. More recently, Balfour and Jones (12) have reported similar studies using gel filtration to monitor the proportion of radiolabeled-IgG bound to placental membrane preparations.

Although a variety of systems existed for measuring IgG binding to placental Fc-receptors, we wanted to develop a simple assay that would allow us to detect FcR activity present on purified plasma membrane vesicles. Since our principal goal is to purify the FcR, the presence of this activity on the membranes had to be confirmed if the vesicles were to be used as a starting point for the isolation procedure.

Plasma membrane vesicles, isolated from fresh placental tissue, were capable of agglutinating IgG-sensitized red blood cells, and this agglutination could be inhibited by monomeric IgG in a dose-dependent fashion. This phenomenon formed the basis of an assay that was used to investigate the characteristics of binding to the FcR. The results were consistent, in many cases, with previous findings and provided further insight as to the nature of the interaction between IgG and the receptor(s) present in human placenta.

MATERIALS AND METHODS

Preparation of plasma-membrane vesicles. Placentae were obtained within 30 min of delivery and used to prepare plasma membranes according to the method of Snary *et al.* (13) with some minor modifications. Trophoblastic tissue was dissected from fetal membranes and minced thoroughly. The tissue was washed in three separate volumes of ice-cold 0.15 M NaCl, 10

Received for publication August 13, 1979.
Accepted for publication October 11, 1979.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported by Grant MT 4259 from the Medical Research Council of Canada.
[2] Supported by a grant from the National Research Council of Canada under the terms of the Canada-France Exchange Agreement.

[3] Abbreviations used in this paper: FcR, Fc receptor; Ig, immunoglobulin; γ, heavy chain of IgG; L, light chain of IgG; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.

mM Tris HCl buffer, pH 7.4 (TBS) and filtered through stretched muslin. The washed tissue was disrupted by passage through a tissue press of the type described by Snary *et al.* (13), and the homogenate was filtered through muslin to remove fibrous connective tissue. The filtrate was collected and centrifuged at 4000 × G for 10 min at 4°C. The supernatant was centrifuged at 20,000 × G for 30 min to yield a crude microsomal pellet. This pellet was further fractionated on a discontinuous sucrose density gradient as follows. The pellet was resuspended in TBS and adjusted to 36% (w/w) sucrose, by using refractive index, and overlayed with 25% (w/w) sucrose in TBS. After centrifugation at 70,000 × G for 12 hr at 4°C, the microsomal fraction resolved into an interfacial band and a pink pellet. The interfacial band representing the plasma-membrane fraction was harvested, washed twice in TBS, and adjusted to between 5 and 10 mg protein/ml in the same buffer. The pink pellet represented an ER-enriched fraction and was usually discarded. It was necessary to use the membranes immediately because storage at 4°C led to a progressive loss in FcR activity. However, plasma membranes recovered from the gradients could be snap-frozen in sucrose and subsequently stored at −57°C without loss of activity. Before use, these membranes were rapidly thawed and washed, and their concentration was adjusted as described above.

Sensitization of human erythrocytes with anti-D. Human erythrocytes coated with human antibody were prepared by using red cells obtained from either of two blood group A Rh(D)-positive donors. One volume of the packed, washed cells was incubated at 37°C for 1 hr with 4 volumes of 0.9% saline and 4 volumes of a 1:20 dilution, in saline, of full strength human Rh_o(D)-immune globulin (Connaught Laboratories, Toronto). After incubation, the cells were washed four times in saline to remove unbound protein and then taken up in 40 volumes of saline (a 2.5% solution of red cells). Unsensitized control cells were prepared by using saline.

Agglutination assay for the detection of Fc receptors. Plasma membrane vesicles, either freshly prepared from a single placenta or from a stock supply of membranes pooled from two to three placentae that had been snap-frozen in hypertonic sucrose, were adjusted to a concentration of between 5 and 10 mg protein/ml with TBS. In a typical experiment, 50 μl of the membrane suspension were added to a small test tube followed by 100 μl of TBS, pH 7.4, and 50 μl of the sensitized (or unsensitized) red blood cell suspension. If an inhibition study was being done, the vesicles were preincubated with 100 μl of the inhibiting protein in TBS for 10 min at 37°C before the addition of the RBC. Control samples having red blood cells, but no vesicles, were included with all experiments. To test for heterophilic antibodies or anti-IgG antibodies, sensitized red cells were incubated with the various immunoglobulin preparations at the highest and lowest concentrations used. No agglutination was observed. All samples were at the same final volume. The samples were mixed well and incubated at 37°C for approximately 1 hr. Each sample was spun down for exactly 1 min at 400 × G. A small portion of the red cell button was transferred to a microscope slide by using a Pasteur pipette. The slide was gently rocked to spread the cells and the agglutination was scored at 100× magnification in a Zeiss light microscope. The samples were coded and agglutination was evaluated by using the following scoring system (see also Fig. 1):

−ve, all free red blood cells, no agglutinates;
+1, clumps of 6 to 10 cells, many free cells still apparent;
+2, clumps of 15 to 30 cells, fewer free cells;
+3, large islands and sheets of cells, clumps may cover one-third to one-half of the viewing field, few free cells detectable;
+4, massive sheets and clumps of agglutinated cells, clumps may cover most of the viewing field, no free cells.

Intermediate agglutination was scored accordingly.

Both within a given experiment and by using preparations from different placentae, the results were found to be highly reproducible. However, the absolute concentration of protein required to inhibit agglutination was dependent on the degree to which the indicator cells were sensitized with anti-D antibodies (measured by the standard antiglobulin test). For the majority of experiments, red cells from one individual, which were strongly sensitized with anti-D immune globulins, were used. However, in the species-specificity studies, the sensitivity of the assay was increased by using cells obtained from a second donor, which did not sensitize as strongly. With these cells, lower amounts of IgG were required to inhibit agglutination.

Proteins. Proteins corresponding to the four human IgG subclasses were isolated from the plasma of patients with multiple myeloma. Purified IgG was obtained by ammonium sulfate precipitation followed by chromatography on DEAE-cellulose (14). Human IgA1, IgM, and rabbit IgG were kindly provided by Dr. B. J. Underdown. IgG1 (DOB), which has a deletion corresponding to the entire hinge region (residues 216 to 230), was provided by Dr. D. R. Davies. The preparation of bovine, murine, sheep, goat, dog, and guinea pig IgG has been described elsewhere (15).

Fab and Fc fragments from human IgG1 were prepared by solid-phase tryptic digestion as described by Ellerson *et al.* (16). Fc fragments from the other IgG subclasses were prepared by papain (Worthington, Freehold, N. J.) digestion in the presence of 1 mM cysteine (for IgG2 and 4) and subsequently purified by ion-exchange and gel chromatography. pFc′ was prepared by peptic digestion of IgG1 according to the procedure of Turner *et al.* (17).

The interchain disulfide bonds of IgG and Fc were reduced with 10 mM dithioerythrytol (Sigma Chemical Co., St. Louis, Mo.) in 0.5 M Tris HCl, pH 8.0. After 30 min at room temperature, the solution was made 25 mM in iodoacetamide and kept in the dark for 15 min. Excess reagents were removed by exhaustive dialysis into TBS. The extent of reduction was determined by SDS-polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (18).

The purity of all proteins and fragments was assessed by SDS-PAGE, immunoelectrophoresis, and double diffusion in agar against polyvalent and monospecific antisera. Proteins were dialyzed against TBS and stored in aliquots at −20°C. Protein concentrations were determined spectrophotometrically at 280 nm by using the following values of $A_1^{1\%}{}_{cm}$: IgG, IgA, 14:0; IgM, 12.5; Fc, 13.8; pFc′, 15.3; nonhuman IgG, 14.0.

Other techniques. 5′-nucleotidase activity was measured by the method of Aronson and Touster (19). The concentration of membrane protein was determined by using a modification of the Lowry technique, which employs sodium deoxycholate to solubilize the proteins (20).

RESULTS

Isolation of plasma membrane vesicles. The purification of placental plasma membrane vesicles from intact trophoblastic tissues was monitored by transmission electron microscopy and by SDS-PAGE. A representative gel depicting the changing protein composition as a function of purification, is shown in Figure 2.

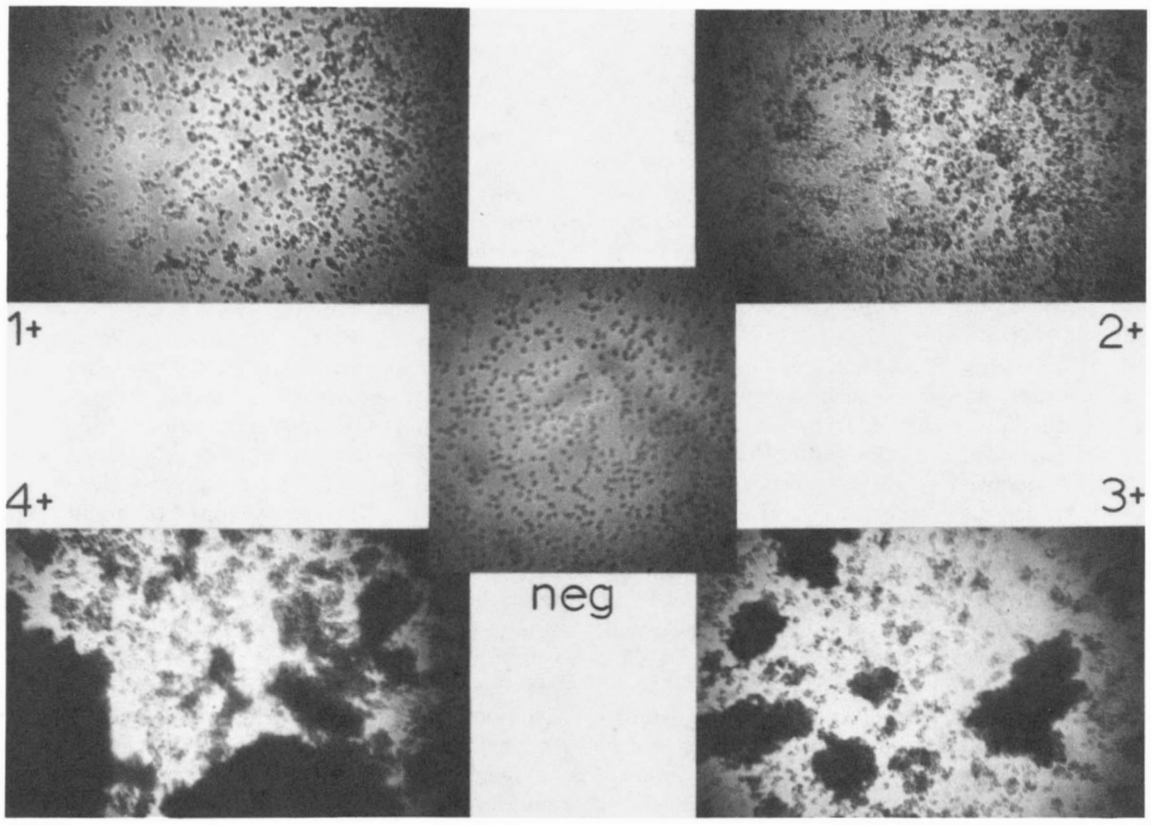

Figure 1. Scoring system for agglutinations. Red cells from donor 1, sensitized with human anti-Rh_o $(D)_1$ were incubated for 1 hr at 37°C with increasing amounts of plasma membrane vesicles. The agglutinations pattern was viewed by using a light microscope and evaluated by the scoring system indicated.

a) No membrane protein added—agglutination score = 0
b) 100 μg of membrane protein added—agglutination score = 1+
c) 850 μg of membrane protein added—agglutination score = 2+
d) 1200 μg of membrane protein added—agglutination score = 3+
e) 2400 μg of membrane protein added—agglutination score = 4+

In order to ensure that we were dealing with a preparation enriched in plasma-membrane vesicles, the 5′-nucleotidase activity in each of the fractions was monitored. 5′-nucleotidase is a well-characterized plasma membrane-associated ectoenzyme frequently used as a specific marker for this organelle (21). Routinely, a 20-fold enrichment was obtained with a corresponding yield of 20 to 25% based on the homogenate. These studies also indicated that maximal yields of plasma membrane vesicles could be obtained if fresh rather than frozen tissue was used. For this reason, all subsequent experiments were carried out with fresh tissue.

Agglutination of IgG-sensitized red blood cells by plasma-membrane vesicles. The agglutination observed when IgG-sensitized red blood cells were incubated with plasma-membrane vesicles was found to be a function of the amount of membrane protein used in the incubation (Fig. 3). The small amount of agglutination observed with high concentrations of endoplasmic reticulum was consistent with the level of plasma membrane contamination indicated by the 5′-nucleotidase assay (approximately 15 to 20%). No agglutination was observed when unsensitized red cells were used in the incubation.

Inhibition of agglutination by human immunoglobulin classes and subclasses. IgG-sensitized red blood cells were incubated with a constant amount of plasma-membrane vesicles, sufficient to give a strong agglutination pattern (final concentration ≃ 2.5 mg of membrane protein/ml), and increasing amounts of IgG1, IgA, or pentameric IgM. Only IgG1 was able to inhibit the agglutination reaction (Fig. 4). The ability of the four subclasses of human IgG to inhibit the assay was also tested, and this study indicated that IgG1 and IgG3 are more effective inhibitors than IgG2 and IgG4[4] (Fig. 5*A*). The amount of any immunoglobulin required to completely inhibit agglutination did not vary significantly in different experiments with red cells from one individual sensitized by the standard procedure outlined in the *Materials and Methods* section.

Species specificity of the binding to the placental receptor. To gain information on the emergence and degree of conservation of the structural elements required for the interaction of IgG with the placental FcR, the ability of purified IgG from various species to inhibit the agglutination system was investigated (Fig. 6*A*, *B*). Since only limited amounts of these immunoglobulins were available, red cells from a second individual were used in this series of experiments. These red cells did not sensitize as strongly with the standard amount of anti-D antibodies and therefore the agglutination reaction could be inhibited by lower concentrations of protein than were normally required. The results were found to be reproducible by using this system, and marked differences in the ability of the immunoglobulins from different species to inhibit agglutination

[4] The possibility that the low level of inhibition observed for IgG2 and IgG4 was due to contamination with IgG1 was excluded by analyzing high concentrations of these proteins (7 mg/ml) in double diffusion against an antiserum monospecific for IgG1.

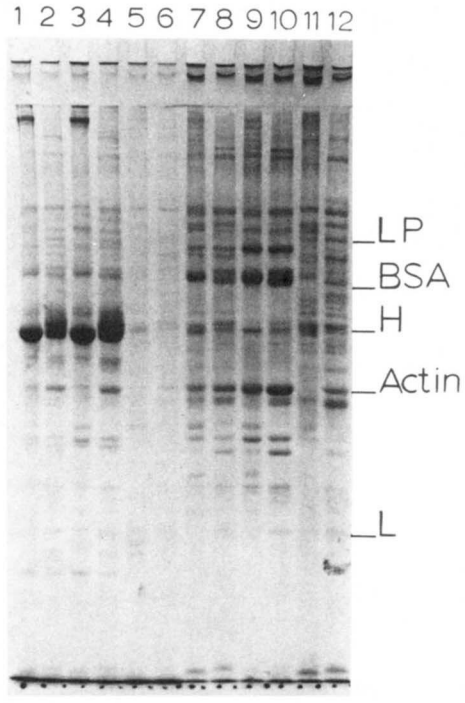

Figure 2. Purification of placental plasma membrane vesicles as seen by SDS-PAGE. At each stage in the purification procedure, samples were saved to run on SDS-PAGE (10%) and later stained with Coomassie Blue. Approximately 45 μg of protein was used in each case.

Slot No.	*Sample*
1	Filtrate
2	Filtrate (R)[a]
3	4,000 × G supernatant
4	4,000 × G supernatant (R)
5	4,000 × G pellet
6	4,000 × G pellet (R)
7	20,000 × G pellet
8	20,000 × G pellet (R)
9	Plasma membrane
10	Plasma membrane (R)
11	Endoplasmic reticulum
12	Endoplasmic reticulum (R)

[a] R = reduced with β-mercaptoethanol.

Molecular weight markers were as follows:
LP = lactoperoxidase, m.w. = 92,500
BSA = bovine serum albumin, m.w. = 67,000
H = heavy chain from human IgG1, m.w. = 52,000
Actin = purified rabbit muscle Actin, m.w. = 42,000
L = light chain from human IgG1, m.w. = 22,000

were observed. The order was human IgG1 > rabbit IgG, mouse Ig2a, or guinea-pig IgG2, which, in turn, was >guinea-pig IgG1, mouse IgG1, mouse Ig2b or dog IgG. IgG from sheep, goat, and cow did not inhibit agglutination over the concentration range tested.

Inhibition of agglutination by IgG fragments. The strong agglutination pattern normally observed with plasma-membrane vesicles could be inhibited by adding increasing amounts of Fc to the reaction mixture (Fig. 7*A*). Fab, in contrast, was not able to inhibit agglutination. The Fc specificity on the reaction was further confirmed by the observation that red cells, sensitized with the $F(ab')_2$ fragment of anti-D antibodies (a gift of Dr. D. G. Romans) could not be agglutinated by plasma-membrane vesicles. A subfragment of Fc corresponding to the entire Cγ3 domain (pFc′) was not inhibitory even at concentrations 500 times in excess of those required for inhibition by Fc. The inhibitory ability of Fc fragments derived from the four human IgG subclasses was also studied (Fig. 5*B*). This experiment indicated that the relative inhibitory potency of the Fc fragments paralleled that exhibited by the intact parent proteins.

Effect of reduction and alkylation on the binding of IgG and Fc fragments. Further information on the structural requirements for mediating inhibition was obtained by testing the ability of IgG1 and $Fc\gamma_1$, which had been reduced and alkylated, to inhibit the agglutination reaction. As shown in Figures 7*A* and *B*, only limited inhibition was seen with both reduced and alkylated Fc and IgG. The importance of the hinge region was confirmed when the protein DOB was tested as an inhibitor. This IgG1 myeloma protein has an internal deletion corresponding to the entire hinge region (residues 216–230) and hence lacks the inter-heavy chain disulfide bonds (22). This protein showed a markedly reduced ability to inhibit agglutination that was unchanged when the protein was reduced and alkylated.

DISCUSSION

For a variety of reasons, systems that have previously been used to study the binding of IgG to the placental FcR may not accurately reflect the *in vivo* situation. Many of these studies involve altering the ligand by either radioactive or fluorescein

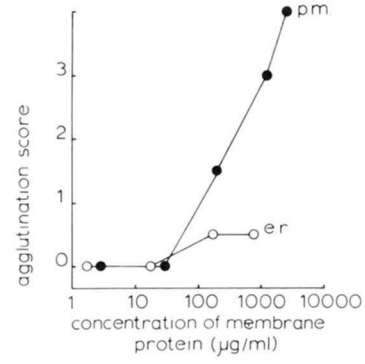

Figure 3. A comparison of FcR activity in purified plasma membranes (●——●) and endoplasmic reticulum (○——○)-enriched vesicles. Aliquots of each preparation were incubated for 1 hr at 37°C with a 2.5% solution of sensitized red blood cells (donor 1). Agglutination was scored as discussed previously.

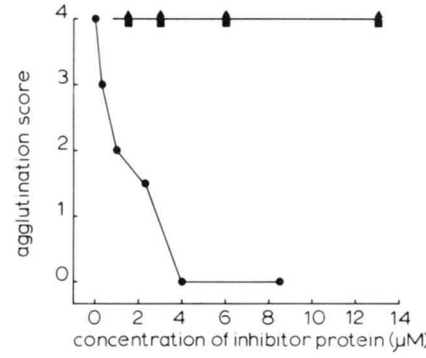

Figure 4. Inhibition of agglutination by IgG1 (●——●), IgA (▲——▲), or IgM (■——■). Aliquots of plasma membrane vesicles containing 400 μg of membrane protein were incubated for 1 hr at 37°C with a 2.5% solution of sensitized red blood cells (donor 1) and increasing concentrations of the proteins indicated. Agglutination was scored by the standard procedure.

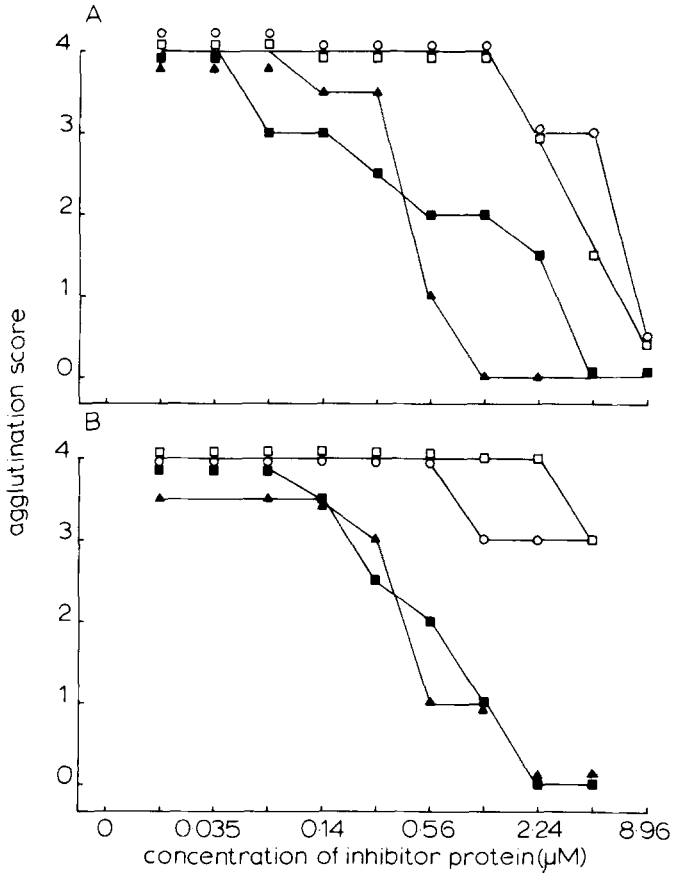

Figure 5. Ability of human subclasses to inhibit agglutination. Aliquots of plasma membrane vesicles containing 500 μg of membrane protein at 37°C with a 2.5% solution of sensitized red blood cells (donor 1) and increasing amounts of the proteins indicated. Agglutination was scored as discussed previously. *A*, IgG1 (■——■), IgG2 (□——□), IgG3 (▲——▲) and IgG4 (○——○). *B*, $Fc\gamma_1$ (■——■), $Fc\gamma_2$ (□——□), $Fc\gamma_3$ (▲——▲) and $Fc\gamma_4$ (○——○).

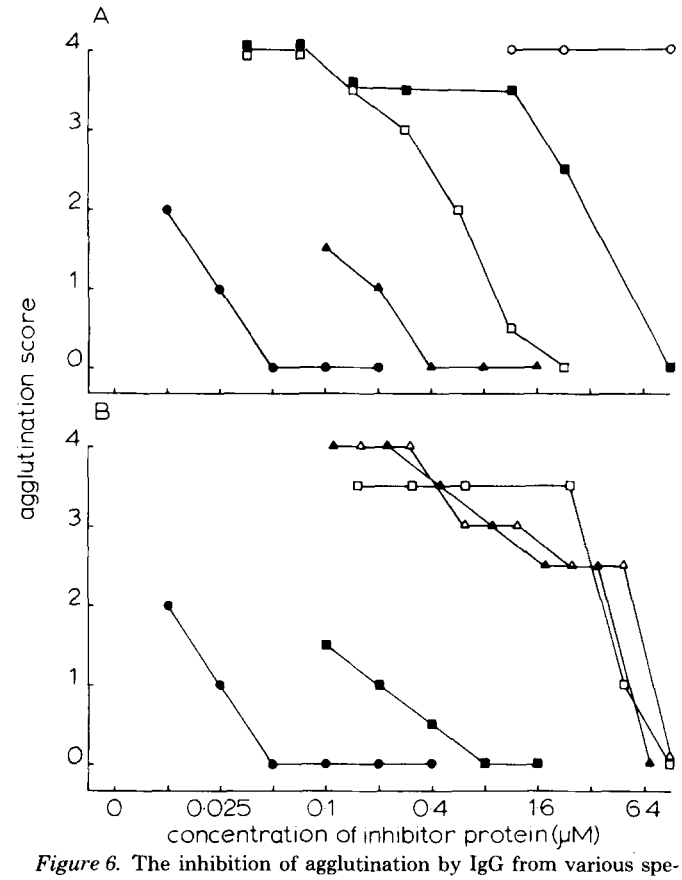

Figure 6. The inhibition of agglutination by IgG from various species. Aliquots of plasma membrane vesicles containing 500 μg of membrane protein were incubated at 37°C for 1 hr with a 2.5% solution of sensitized red blood cells (donor 2) and increasing amounts of the proteins indicated. Agglutination was scored in the standard manner. *A*, human IgG1 (●——●), rabbit IgG (▲——▲), guinea pig IgG1 (■——■), guinea pig IgG2 (□——□), goat, bovine, or sheep IgG (○——○). *B*, human IgG1 (●——●), murine IgG1 (▲——▲), murine IgG2A (■——■), murine IgG2B (□——□), and dog IgG (△——△).

labeling, and one must assume that this modification is without effect on the Fc effector function under study. Other studies have involved cryostat sectioning in which the tissue must be frozen, embedded, and mounted before binding studies can be carried out. Matre and Haugen (23) have recently noted that after such treatment, only limited areas of the sections express FcR activity. When cell preparations have been used, these were obtained by first treating the tissue with trypsin. Our own findings (to be published) are that proteolytic digestion of placental membranes can lead to very strong binding of IgG that is no longer uniquely Fc-mediated. Even those assay systems that make use of membrane preparations, including our previous work, are compromised by the fact that the preparations are significantly contaminated with organelles other than the plasma membrane. By using purified plasma-membrane vesicles obtained from fresh placental tissue and unmodified IgG in the assay system described, we hoped to circumvent some of these problems.

Using a tissue press similar in design to that of Snary *et al.* (13), we were able to obtain a reasonable and reproducible yield of purified plasma-membrane vesicles. Snary *et al.* (13) have reported that with this technique, the plasma membranes obtained were not significantly contaminated with nuclei, mitochondria, endoplasmic reticulum, or entrapped cytosol. By following the 5′-nucleotidase activity in the various fractions, we could establish that the samples used in the agglutination assays were substantially enriched with plasma membranes.

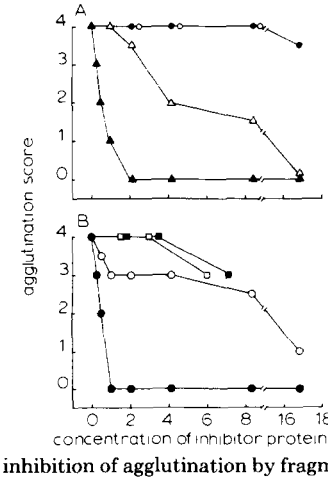

Figure 7. A, inhibition of agglutination by fragments of human IgG1. Increasing amounts of Fc (▲——▲), Fab (●——●), pFc′ (○——○) and Fc which had been reduced and alkylated (△——△) were incubated with a 2.5% solution of sensitized red blood cells (donor 1) and aliquots of plasma membrane vesicles containing 540 μg of membrane protein. Agglutination was scored in the standard manner. *B*, Inhibition of agglutination by native and reduced human IgG1 and DOB, a hinge-deleted human IgG1. Aliquots of plasma membrane protein were incubated for 1 hr at 37°C with a 2.5% solution of sensitized red blood cells (donor 1) and increasing amounts of human IgG1 (●——●), reduced and alkylated IgG1 (○——○), DOB (■——■), and reduced and alkylated DOB (□——□).

We have assumed that the Fc receptor relevant to materno-fetal transmission of IgG must be present on the apical plasma membrane of the syncitiotrophoblast. This assumption is supported by the work of several groups (6, 10, 24). However, Matre *et al.* (6), by using the red blood cells sensitized with either human or rabbit IgG, found Fc receptors on the apical surface of the fetal capillary endothelial cells. Wood *et al.* (25) also using rabbit IgG-sensitized red blood cells, reported a receptor present on many of the stromal cells and in particular on the Hoffbauer cells. In contrast, Jenkinson *et al.* (10) have reported that the majority of cells in placental tissue suspensions forming rosettes with human IgG-sensitized red blood cells are derived from the syncitiotrophoblast and although macrophages are present, these are few in number and contribute little to the total rosette-forming population. The differences remain unresolved and may reflect the fact that IgG from different species were used in the detector systems.

Since the vesicles used in our agglutination system may contain plasma membrane from these other cell types as well as from the syncitiotrophoblast, we cannot be certain that we have a homogeneous population of receptors. One indication that the FcR being studied is associated with the syncitiotrophoblast comes from the fact that plasma membrane vesicles that have been passed over a Con A-Sepharose column do not show any decrease in FcR activity (data not shown). Johnson and Falk (26, and references therein) have shown that unlike endothelial and Hoffbauer cell membranes, syncitiotrophoblast does not bind to Con A. Some of the available data would suggest that the FcR present on syncitiotrophoblast cells have a high affinity for monomeric IgG, whereas those present on macrophages and endothelial cells interact more strongly with aggregated IgG (6, 11, 24, 25).

The possibility of using a classical rosette-type detector system with the plasma membrane vesicles did not at first seem applicable because of the diameter of the vesicles relative to that of the red blood cells (i.e., 200 nm average diameter compared with 7000 nm for the red cell). Upon reconsideration, however, it was decided that if vesicles displaying active receptor molecules on their outer surface were present, such vesicles ought to be able to cross-link IgG-sensitized red blood cells into a three-dimensional lattice. This would be observable as an agglutination pattern either with the naked eye or with a light microscope. This hypothesis was tested and confirmed. The ability of the various fractions to cause agglutination of the sensitized red blood cells was found to correlate with their 5′-nucleotidase activity implying that the Fc receptor was plasma membrane-associated.

The inhibition studies indicate clearly that the agglutination observed is the manifestation of an Fc-mediated interaction. Furthermore, the fact that neither IgA nor IgM can inhibit the interaction indicates that the phenomenon is due to a class-selective receptor consistent with *in vivo* studies (see 27, and references therein). Experiments with the various subclasses of human IgG support our earlier studies on the placental receptor. If one assumes that binding to the receptor is a prerequisite for transport, these data would also support those *in vivo* findings that suggest that IgG1 and IgG3 are preferentially transferred over IgG2 and IgG4 (27, 28). This subclass specificity must result from subtle amino acid substitutions since the Fc sequences of IgG1 and IgG2 have only six amino acid interchanges within the Cγ2 domain and one in Cγ3. The sequences of IgG1 and IgG4 have 13 interchanges in the Cγ2 domain and three in Cγ3 (29).

Information on the nature of the binding site was gained from studies with reduced and alkylated IgG and Fc. Breaking the interchain disulfide bonds was found to greatly reduce the ability of these molecules to inhibit agglutination. The importance of the hinge-region disulfides was further emphasized by the fact that a human IgG1 molecule (DOB), which lacks the entire hinge region, also had a markedly reduced ability to bind to the FcR.

These results are inconsistent with our earlier report that reduced and alkylated Fc had the same affinity for the receptor as the intact fragment. The difference between these two results could reflect the fact that crude membrane preparations, containing other organelles, may exhibit FcR activity other than that expressed on plasma-membrane vesicles. It is interesting to note that others working in this laboratory have found that reduction and alkylation of Fc does not alter its ability to bind to human monocytes (D. Foster and R. H. Painter, to be published). Since these cells are often considered to be macrophage precursors, the discrepancy in our results may be because more macrophage membranes are present in the crude preparation than in the plasma membranes used here, prepared by using a different method of tissue disruption.

Mild reduction and alkylation of either IgG or Fc affects the spatial relationship (and relative mobility) of the Cγ2 domains by removing the covalent constraints imposed by the hinge region disulfides (30). In contrast, Cγ3 domains are held together by multiple noncovalent interactions, and it is assumed that reduction of the hinge region disulfides would do little to alter the spatial relationship between these domains. This would, therefore, tend to exclude the possibility that the Cγ3 domains are in themselves the location of the binding site. To further investigate this point, isolated paired Cγ3 domains, in the form of pFc′, were tested for the ability to inhibit agglutination. CD spectra in the far ultraviolet region were measured and indicated that the molecule had not lost significant structure in the course of purification (data not shown). The finding that this fragment could not inhibit agglutination strongly suggests that the binding site is not restricted to the Cγ3 domains.

The observation that Fc fragments prepared from the four IgG subclasses follow the same order of inhibitory potency as the parent molecules is also of significance in a discussion of the possible location of the binding site. Isenman *et al.* (31) have reported that although IgG4 is unable to fix the C1 component of complement, $Fc\gamma_4$ has activity comparable to that of either $Fc\gamma_1$ or IgG1. The authors concluded that quaternary interactions may exist between Fab and Fc in the intact IgG4 molecule and that these interactions modulate the activity of the C1 binding site on Cγ2. [The C1 binding site had already been localized to Cγ2 (32, and references therein).] Furthermore, since the modulation was quite possibly a simple steric blocking of the C1 binding site by Fab, it was argued that the binding site was probably located near the N-terminus of the Fc fragment, where close apposition of C_L and Cγ2 would be expected to occur.

Since in binding to the placental FcR no significant difference in activity could be detected between parent IgG subclass molecules and their corresponding Fc fragments, our data would be consistent with a binding site more C-terminal than the C1 binding site. Because our experiments rule out a site exclusively on Cγ3, this would leave most of the surface of the Cγ2 domain(s) and a site formed through interaction of Cγ2 and Cγ3 as possible locations for interaction with the placental FcR. We have previously reported that isolated Cγ2 domains do not bind even to the crude placental membrane preparations and therefore feel that the latter location is more likely. The possibility of a shared binding site has been suggested for the binding of

IgG to cytotoxic K cells (33), activated murine T cells (34), rat intestinal epithelial cells (35), murine macrophage-like cells (36), and human granulocytes (37). It is possible that the hinge sequence may determine the angular orientation of the Cγ2 domains and consequently the shape of the Cγ2-Cγ3 junction. If a shared binding site is involved, this could explain why altering the hinge by reduction, deletion, or in the different subclasses has such a marked effect on the binding activity.

Further information on the structural requirements for binding to the placental receptor was obtained by studying the ability of IgG from a variety of species to inhibit the agglutination assay. The mode of transport of passive immunity from mother to young relates to the species, which in turn correlates with their phylogenetic position. In man, the transfer occurs entirely before birth across the placenta. In guinea pig and rabbit, this transfer also occurs before birth, but in these cases, the yolk sac is involved (5, 38). In rat and mouse, antibodies are transported both pre- and post-natally by yolk sac and gut (39, 40). Cats, dogs, and ferrets show limited transfer *in utero*, with the bulk of the transfer occurring in the first 10 days after birth (41). At the far extreme, cows, sheep, goat, horse, pig, and oxen show no prenatal transfer, with transport occurring entirely via the gut after birth (42).

Our results confirm and extend the findings of Matre and Haugen (23) that IgG from species with prenatal transport will bind to the FcR of human placental tissue. In contrast, IgG from animals with no prenatal transport of immunoglobulins do not inhibit the binding of sensitized red blood cells to plasma-membrane vesicles. Our results also indicate that where different subclasses of immunoglobulins within a given species were available for testing, these did not exhibit the same ability to bind to the placental receptor. This further complicates the interpretation of data where red cells sensitized with nonhuman IgG are used in the detector system.

We have previously studied the requirements for IgG binding to the FcR on the murine macrophage-like cell line P388D_1 and found that except for guinea pig, the species specificity of that interaction is similar to the results obtained with placental tissue (15). The specificity shown by the two receptors for the human IgG subclasses is also very similar. This, along with the fact that neither pFc′ nor Cγ2 could bind to the FcR on P388D_1 cells, implies that the receptor on these cells and on human placenta may have similar structural requirements for IgG binding. The suggestion that the site on IgG responsible for binding to these receptors is contributed to by both Cγ2 and Cγ3 is further supported by the fact that Protein A from *Staphylococcus aureus* shows the same specificity for binding [murine, rabbit, canine, guinea pig, and human IgG bind to Protein A whereas sheep, goat, and bovine IgG bind weakly or not at all (43)] and is known to bind to an inter-domain site (44). Thus, a spatially related site on IgG may be involved in binding to all three.[5] The significance of the fact that the evolutionary appearance of this site parallels the appearance or maintenance of a prenatal transport system remains unclear.

Acknowledgments. We would like to thank the staff of the obstetrical floors of the Toronto General, Mount Sinai, and Women's College Hospitals for making the placentae available. We would also like to thank Dr. D. G. Romans and Dr. D. E. Isenman for their helpful discussions.

[5] It would be of interest to determine if the monovalent fragment B of Protein A were able to prevent the binding of IgG or Fc to the FcR on placenta or P388D_1 cells.

REFERENCES

1. Wild, A. E. 1974. Protein transport across the placenta. Reprinted from Symposia of the Society for Experimental Biology. No. XXVIII. Transport at the Cellular Level. Cambridge University Press.
2. Gitlin, D., J. Kumate, J. Urrusti, and C. Morales. 1964. The selectivity of the human placenta in the transfer of plasma proteins from mother to fetus. J. Clin. Invest. 43:1938.
3. Nöschel, H., A. Stelzner, J. H. Seewald, and G. Klinger. 1975. Untersuchungen zur plazentapassage von virus-antiliorgern und immunglobulinen an der in vitro perfundierten menschlichen plazenta. Zentralblatt fur Gynakologie, Heft. 3, 97:180.
4. Borner, P., H. Deicher, K. Heide, and J. Reinecke. 1973. Evidence for the participation of the Fc portion of immunoglobulin G in maternofoetal immunoglobulin transfer in the human. Int. Congress Immunology in Obstetrics and Gynaecology, p. 272.
5. Brambell, F. W. R. 1966. The transmission of immunity from mother to young and the catabolism of immunoglobulins. Lancet 2: 1087.
6. Matre, S., and P. M. Johnson. 1977. Multiple Fc receptors in the human placenta. Acta Path. Microbiol. Scand. Sect. C, 85:314.
7. Johnson, P. M., P. Trenchev, and W. P. Faulk. 1975. Binding of complexed immunoglobulin by stromal endothelial cells. Clin. Exp. Immunol. 22:133.
8. Johnson, P. M., W. P. Faulk, and A. C. Wang. 1976. Immunological studies of human placentae: subclass and fragment specificity of binding of aggregated IgG by placental endothelial cells. Immunology 31:659.
9. Moskalewski, S., W. Ptak, and Z. Czarnik. 1975. Demonstration of cells with IgG receptors in human placenta. Biol. Neonate 26:268.
10. Jenkinson, E. J., W. D. Billington, and J. Elson. 1976. Detection of receptors for immunoglobulin on human placenta by EA rosette formation. Clin. Exp. Immunol. 23:456.
11. McNabb, T., T. Y. Koh, K. J. Dorrington, and R. H. Painter. 1976. Structure and function of immunoglobulin domains. V. Binding of immunoglobulin G and fragments to placental membrane preparations. J. Immunol. 117:882.
12. Balfour, A. H., and E. A. Jones. 1978. Properties of receptors for IgG on human placental cell membranes. Int. Arch. Allergy Appl. Immunol. 56:435.
13. Snary, D., F. Woods, and M. J. Crumpton. 1976. Disruption of solid tissue for plasma membrane preparation. Analyt. Biochem. 74:457.
14. Sober, H. A., and E. A. Peterson. 1958. Protein chromatography on ion exchange cellulose. Fed. Proc. 17:1116.
15. Haeffner-Cavaillon, N., M. Klein, and K. J. Dorrington. 1979. Studies on the Fc receptor of the murine macrophage-like cell line P388D_1. I. The binding of homologous and heterologous immunoglobulin G. J. Immunol. 123:1905.
16. Ellerson, J. R., D. Yasmeen, R. H. Painter, and K. J. Dorrington. 1976. The structure and function of immunoglobulin domains. III. Isolation and characterization of a fragment corresponding to the Cγ2 homology region of human immunoglobulin G. J. Immunol. 116:510.
17. Turner, M. W., H. H. Bennich, and J. B. Natvig. 1970. Pepsin digestion of human G myeloma proteins of different subclasses. I. Immunochemical investigations of the products of peptic digestion. Clin. Exp. Immunol. 7:627.
18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680.
19. Aronson, N. N., and O. Touster. 1974. Isolation of rat liver plasma membrane fragments in isotonic sucrose. Methods Enzymol. 31:90.
20. Campbell, P. N., and J. R. Sargent. 1967. *In* Techniques in Protein Biosynthesis. Academic Press, New York. P. 22.
21. Yin-Tak Woo, and J. F. Manery. 1975. 5′-Nucleotidase: an ectoenzyme at frog skeletal muscle. Biochem. Biophys. Acta 397:144.
22. Steiner, L. A., and A. G. Steinberg. 1979. The crystallizable myeloma protein DOB has a hinge region deletion. Fed. Proc. 38, No. 1, Abstract 4094.
23. Matre, R., and A. Haugen. 1978. The placental Fc receptors studied

using immune complexes of peroxidase. Scand. J. Immunol. 8:187.
24. Wood, G., J. Reynard, E. Krishnan and L. Racela. 1978. Immunobiology of the human placenta: IgG Fc receptors in trophoblastic villi. Cell. Immunol. 35:191.
25. Wood, G., J. Reynard, E. Krishnan, and L. Racela. 1978. Immunobiology of the human placenta: localization of macrophages, in vivo bound IgG and C3. Cell. Immunol. 35:205.
26. Johnson, P. M., and W. P. Faulk. 1976. Immunological studies of human placentae: lectin binding to villous stroma and to trophoblastic basement membrane. Clin. Exp. Immunol. 24:435.
27. Virella, G., M. Nunes, and G. Tamagnini. 1972. Placental transfer of human IgG subclasses. Clin. Exp. Immunol. 10:475.
28. Wang, A. C., W. P. Faulk, M. A. Stuckey, and H. H. Fudenberg. 1970. Chemical differences of adult, fetal, and hypogammaglobulinemic IgG immunoglobulins. Immunochemistry 7:703.
29. Dayhoff, M. O. 1972. *In* Atlas of Protein Sequence and Structure. Vol. 5. National Biomedical Research Foundation. pp. D229–D274.
30. Romans, D. G., C. A. Tilley, M. C. Crookston, R. E. Falk, and K. J. Dorrington. 1977. Conversion of incomplete antibodies to direct agglutinins by mild reduction: evidence for segmental flexibility within the Fc fragment of immunoglobulin G. Proc. Natl. Acad. Sci. 74:2531.
31. Isenman, D. E., K. J. Dorrington, and R. H. Painter. 1975. The structure and function of immunoglobulin domains. II. The importance of interchain disulfide bonds and the possible role of molecular flexibility in the interaction between immunoglobulin G and complement. J. Immunol. 114:1726.
32. Yasmeen, D., J. R. Ellerson, K. J. Dorrington, and R. H. Painter. 1976. The structure and function of immunoglobulin domains. IV. The distribution of some effector functions among the Cγ2 and Cγ3 homology regions of human immunoglobulin G. J. Immunol. 116: 518.
33. Michaelsen, T. E., F. Wisloff, and J. B. Natvig. 1975. Structural requirements in the Fc region of rabbit IgG antibodies necessary to induce cytotoxicity by human lymphocytes. Scand. J. Immunol. 4: 71.
34. Klein, M., C. Neuport-Sautes, J. R. Ellerson, and W. H. Fridman. 1977. Binding site of human IgG subclasses and their domains for Fc receptors of activated murine T cells. J. Immunol. 119:1077.
35. Guyer, R. L., M. E. Koshland, and P. M. Knopf. 1976. Immunoglobulin binding by mouse intestinal epithelial cell receptors. J. Immunol. 117:587.
36. Haeffner-Cavaillon, N., K. J. Dorrington, and M. Klein. Studies on the Fcγ receptor of the murine macrophage-like cell line $P388D_1$. II. Binding of human IgG subclass proteins and their proteolytic fragments. J. Immunol. 123:1914.
37. Barnett Foster, D. E., K. J. Dorrington, and R. H. Painter. 1978. Structure and function of immunoglobulin domains. VII. Studies on the structural requirement of human immunoglobulin G for granulocyte binding. J. Immunol. 120:1952.
38. Leissring, J. C., and J. W. Anderson. 1961. The transfer of serum proteins from mother to young in the guinea pig. I. Prenatal rates and routes. Am. J. Anat. 109:149.
39. Brambell, F. W. R., and R. Halliday. 1956. The route by which passive immunity is transmitted from mother to foetus in the rat. Proc. R. Soc. B 145:170.
40. Gitlin, J. D., and D. Gitlin. 1974. Protein binding by specific receptors on human placenta, murine placenta and suckling murine intestine in relation to protein transport across these tissues. J. Clin. Invest. 54:1155.
41. Brambell, F. W. R. 1970. The transmission of passive immunity from mother to young. *In* Frontiers of Biology. Vol. 18 North Holland Publishing Co., Amsterdam.
42. Mason, J. H., T. Dalling, and W. S. Gordon. 1930. Transmission of maternal immunity. J. Pathol. Bacteriol. 33:783.
43. Kronwall, G., U. S. Seal, J. Finstad, and R. C. Williams. 1970. Phylogenetic insight into evolution of mammalian Fc fragment of γG globulin using staphylococcal protein-A. J. Immunol. 104:140.
44. Deisenhoffer, J., T. A. Jones, P. Huber, J. Sjodahl, and J. Sjoquist. 1978. Crystallization, crystal structure analysis and atomic model of the complex formed by a human Fc fragment and fragment B of protein A. Hoppe-Seyler's Z. Physiol. Chem. 359:975.