Determination of the neighboring molecule to the FC receptor on human macrophages

Lorri Jean Malinski

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DETERMINATION OF THE NEIGHBORING MOLECULE
TO THE FC RECEPTOR
ON HUMAN MACROPHAGES

By

Lorri Jean Malinski

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ABSTRACT

DETERMINATION OF THE NEIGHBORING MOLECULE TO THE FC RECEPTOR ON HUMAN MACROPHAGES

By

Lorri Jean Malinski

Chemical cross-linking studies have been carried out to investigate near neighbors to the receptor for immunoglobulin G (IgG) on U937 cells both before and after solubilization of the receptor. A cross-linked product with a molecular weight of 350,000 daltons was achieved with OMS on intact and lysed U937 cells, OTBP on intact cells and OSS on cell lysates, with anti-FcRII as the immunoprecipitation reagent. Following cross-linking of the U937 cells with OTBP and analysis by two-dimensional gel electrophoresis the p350 molecule appeared to be a p170 dimer. The p170 molecular has been related to some nonspecificity of the anti-FcRII. These procedures were thus unsuccessful in identifying a neighboring molecule to the U937 cell FcR.

OSS cross-linking of Fc fragments of IgG and Fab fragments of anti-FcRII to the FcR on intact U937 cells was unsuccessful. Lack of success in this regard is more likely attributed to difficulties in OSS usage then to inability to cross-link close-lying molecules to the FcR.

The investigations presented clarify the problems that must be overcome before successful cross-linking can be achieved between the U937 cell FcR and its neighboring molecule(s).
TO TOM

Your love, understanding and encouragement has made this possible
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INTRODUCTION

The cell surface receptor for immunoglobulin G (IgG) on human macrophages and monocytes mediates the triggering of endocytosis. The endocytic process begins with the binding of IgG immune complexes through their Fc units to these receptor sites on macrophages (See Figure 1). The nature of the triggering signal as well as the entire sequence of events of this process are largely unknown. A proper starting point to achieve an understanding of these events is the characterization of the entire Fc binding site which is known as the Fc receptor (FcR) [1]. What is known is that the FcR is an integral membrane protein which travels as a single major heterodisperse band on polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulfate (SDS) with a molecular weight of approximately 72,000 daltons (2). It is believed that this p72 molecule does not make up the FcR in its entirety and that the FcR is a complex of subunits. The objective of this study has been to determine the neighboring molecule to the FcR using cross-linking reagents with the consideration that the close lying molecule may be a subunit of the receptor site. Cross-linking reagents in use were two imidates, dimethyl suberimidate (DMS) and dimethyl-3,3'-dithiobispropionimidate (DTBP), an n-hydroxysuccinimide ester, disuccinimidyl suberate (DSS), as well as a photosensitive reagent N-5-azido-2-nitro-benzoyloxsuccinimide (ANB-NOS).
Figure 1. The four chain structure of an IgG molecule showing both interchain and intrachain disulfide bridges. Indicated are the Fc and Fab fragments. Pepsin digests away most of Fc leaving a molecule, F(ab')2, capable of precipitating antigens because it is bivalent. (Cbh = carbohydrate) (From reference 61.)
SECTION I

BACKGROUND MATERIAL

1.1 Fc RECEPTORS

Fc receptors can be found on the plasma membrane of many different cell types and tissues, including cells of the immune system, platelets and placental tissue. These cells and tissues may contain single or multiple types of receptors as there are specific Fc receptors for each immunoglobulin class and in some cases, subclass. In other words, the FcR for IgG is a separate entity from the IgE FcR and IgG\textsubscript{1} has a different receptor site than IgG\textsubscript{2}. The logic of this arises from the distinct function each immunoglobulin fulfills. For example, the binding of IgE to its FcR on mast cells and basophils is responsible for the explosive degranulation of these cells which leads to the physiological consequences of the allergic response.

1.2 ENDOCYTOSIS

Endocytosis, a collective term for phagocytosis and pinocytosis, is a process by which cells internalize nutrients, toxins, effector molecules (growth factors, hormones, antibodies), enzymes and pathogens. These substances bind specifically or nonspecifically to the cellular plasma membrane (PM) prior to internalization. Ingested material is then enclosed in membrane vacuoles or vesicles. Fusion of these vacuoles with lysosomes or other organelles allows modification of the endocytosed material within a closed compartment.

In primitive organisms phagocytosis and pinocytosis are a means of feeding. In contrast,
The more highly differentiated responses of mammalian polymorphonuclear leukocytes and macrophages are designed to locate and remove antibody coated material by the process of FcR-mediated endocytosis. Bacteria represent one example of material that is removed in this manner. Antigenic material from bacteria is recognized and ingested following interaction with IgG and complement components. IgG and complement facilitate adherence and subsequent phagocytosis by macrophages. The bacteria are then killed and degraded by enzymes contained in the lysosomes and specific granules.

The process by which foreign material is ingested results from an interaction that occurs between membrane receptor protein and components of the cytoskeleton. Filamentous structures within the cytoskeleton are involved in cellular motility, exocytosis, membrane ruffling and subsequently the "zipper phenomena" as illustrated in Figure 2.

During endocytosis a macrophage can rapidly interiorize a large portion of its plasma membrane. Studies on the Fc receptor would not be complete without giving consideration to its fate during this internalization process. Mellman et al. utilized a method for labelling integral membrane proteins of pinocytic vesicles of the murine macrophage and found the presence of the FcR comparable in the pinocytic vesicle and plasma membrane [3]. This indicated to Mellman that the FcR is cycled from the plasma membrane to the pinocytic vesicle and back again. It is not understood what happens to the FcR once a vesicle fuses with a lysosome.

A second concern when studying the FcR is that of macrophage FcR turnover. Synthesis and insertion times for the FcR have been found to be 36 to 55 minutes from pulse chase experiments. Under normal physiological conditions, turnover of the protein was biphasic since 60% was lost with a half time of 13 hours and the remainder with a half life of 80 hours or greater [4].
Figure 2. The formation of a phagosome in a macrophage interacting with IgG-coated red blood cells via the Fc receptor on the macrophage plasma membrane. Diagram shows attachment, engulfment and membrane zipperling, leading to formation of a phagosome. Symbols: O, red blood cells; --, IgG, Fc receptor. [Taken from reference 5].
The FcR mediated endocytic process can be likened to the process by which drugs, hormones and neurotransmitters exert their effect. That is, receptor proteins must transduce information from the external environment into meaningful intracellular signals. To do so, these receptors must perform three distinct functions: 1) recognition of a specific stimulus, 2) transduction of the signal across the plasma membrane and 3) initiation of a response in the cell. Because of this, receptor proteins are assumed to span the entire membrane. The following model for receptor function has been suggested from these assumptions:

1. Specific interaction of the binding domain of the receptor with an Fc region of an immunoglobulin involves the usual molecular complementarity observed in enzyme(substrate and antigen/antibody reactions.

2. Transduction involves conformational changes in the transducer domain. The binding of ligand could cause changes in the individual receptor molecules; or aggregation of identical membrane receptors with subsequent interaction could induce conformational changes.

3. The initiator domain may act through a variety of pathways. There may be direct interaction between the initiator and the cytoskeletal system which initiates or terminates movement, opens or closes specific ion gates, or activates nucleotide cyclases to produce second messengers cyclic AMP (cAMP) or cyclic GMP (cGMP). These second messengers then modulate the activity of soluble or secretory proteins.

1.3 IgE/FcR SYSTEM

An example of a receptor dependent process which follows the model just suggested is the IgE mediated allergic response. Symptoms of hypersensitivity are the result of the interaction of immunoglobulin E with mast cells and/or basophils. Upon primary exposure to an allergen, antigen specific IgE is produced and becomes fixed to mast cells via the FcR. Upon subsequent exposure to the same antigen, an antigen/antibody reaction occurs on the mast cell plasma membrane. This triggers a series of events that leads to the release of several active mediators of hypersensitivity, including
histamine, from mast cells (6).

1.3A Binding Characteristics

Investigations into the structure of the IgE FcR and the mechanism of histamine release have been performed primarily on rat mast cells and the related tumor line, rat basophilic leukemia cells (RBL). The general properties as well as the binding characteristics of the IgE FcR's found on these cells are listed in Table 1. (18)

1.3B FcR Dimerization

The IgE isotype was discovered in the late 1960's by Ishizaka and Ishizaka (7). Their early work with this immunoglobulin focused on identifying the transducer signal for histamine release. Using a ragweed antigen-human antibody system they showed that an antigen bound to two or more IgE antibody molecules could produce a biological reaction such as skin reactivity; whereas a single antibody could not (8). They later observed that responses could also be initiated with aggregated IgE (9). Ishizaka also developed human anti-IgE antibodies and found that they were capable of inducing mediator release from human basophils. Enzymatic digests were made of the anti-IgE antibodies to form (Fab')$_2$ fragments and monomeric Fab (See Figure 1). The (Fab')$_2$ fragments were capable of producing a response while the monomer bound yet could not generate a response (10).

The above findings suggested that a dimeric signal was sufficient to produce a biological reaction. With this theory in mind, Isersky et. al. (11) developed rabbit IgG antibodies directed against the IgE FcR on rat mast cells. These anti-receptor antibodies and their (Fab')$_2$ fragments caused histamine release from the mast cells while monomeric Fab fragments bound but were not active. These studies implicated receptor aggregation as the triggering stimulus. The next step was to determine how many IgE FcR's need
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to interact for the response. IgE antibody was covalently crosslinked into dimers, trimers and higher forms and their effect on normal rat mast cells was investigated [12]. The mast cells were found to release equally well with all the IgE forms. Similar studies on human basophils using covalently cross-linked human IgE antibodies revealed that IgE dimers, trimers and higher oligomers caused histamine release [13]. In this particular study, IgE trimers were found to be more potent than dimers. However, a comparison of the histamine release curves for dimers and trimers showed parallelism as well as similar maxima. Other studies indicate that the human IgE trimers may be more active due to a greater probability of binding [14]. A logical conclusion from the above findings is that the transduction signal in IgE-mediated reactions is FcR dimerization.

1.3.C Biochemical Studies

In order to evaluate the relationship between plasma membrane receptor proteins and the cytoskeleton, biochemical analyses were performed on various cell types following the stimulation of their IgE Fc receptors. One particular area under investigation was the methylation of phospholipids in cell membranes. Hirata and Axelrod [15] discovered that the conversion of phosphatidylserine to phosphatidylethanolamine and then to phosphatidylcholine is facilitated by methyltransferases in the plasma membrane of rat erythrocytes. These same investigators found that rat mast cells stimulated by Concanavalin A (Con A) in the presence of \(^{14}C\)-phosphatidylserine undergo the above sequence of phospholipid methylations [16]. Since Con A is also responsible for histamine release a correlation between phospholipid methylation and histamine release was suspected.
As a result of the above findings Ishizaka studied phospholipid methylation in rat mast cells stimulated by anti-rat basophilic leukemia antibody or anti-RBL. Ishizaka found that [methyl-³H] L-methionine uptake was maximal 15 seconds after cell stimulation with a rapid decline thereafter. Phospholipid methylation was then followed by histamine release and ⁴⁵Ca uptake. Fab' monomers of anti-RBL did not induce these reactions [17]. Further studies by Ishizaka suggested that phospholipid methylation was intrinsic to the opening of Ca⁺² channels for Ca influx and histamine release. Ishizaka also measured intracellular cAMP and found the levels to be increased after receptor bridging. The exact relationship between adenylate cyclase, methyltransferases and IgE receptors could not be clarified although Ishizaka believes it is crucial to this cellular process.

1.3.D IgE FcR Structure

The structure of the high-affinity mast cell receptor for IgE has been clarified. Metzger describes this receptor as having two subunits, alpha (α) and beta (β)[18]. The α chain has a molecular weight of approximately 50,000 daltons [19] while the β chain has a molecular weight of 30,000 daltons [20]. Cleavage of these chains with proteases has delineated two domains in each [α₁α₂β₁β₂] [21]. The α₂ domain appears to be the site of IgE binding since it can not be labelled when IgE is present. The α and β chains are associated noncovalently in a 1:1 ratio. Metzger has actually proposed a topographical model of the subunits of the IgE FcR and this can be seen in Figure 3. [18] This model has not, as yet, been supported by sufficient data. Further support of receptor subunit structure is provided by other receptor systems such as insulin and acetylcholine [22, 23].

The above studies and findings on the IgE FcR serve as a prototype for the research being conducted on the IgG FcR.

1.4 IgG/FcR SYSTEM

1.4A Binding Studies
Figure 3. Schematic representation of receptor for IgE. The horizontal line represents the surface of the outer leaflet of the plasma membrane bi-layer. The hatched areas represent carbohydrate, the black areas sites of proteolytic cleavage. (*) Principal site of surface labeling. (**) The circles represent spheres whose volumes are proportional to their masses. (From reference 18).
The first step of the endocytic process, the binding of monomeric IgG to macrophage FcR sites, has been characterized by radioligand studies (24-26). This characterization was made possible by the development of a method to separate cell bound radioligand from free radioligand. In this manner monomeric IgG was found to bind only for a matter of minutes and with affinity constants \( (k_a) \) of \( 10^6 \) to \( 10^8 \). These \( k_a \)'s are significantly lower than those for IgE.

1.4B FcR dimerization

As with the IgE System, IgGFcR dimerization appears to trigger the respective cell function of endocytosis. The fate of monomeric IgG upon binding to its receptor at \( 0^\circ \text{C} \) is subsequent dissociation from the cell membrane (27). Anderson has determined that the kinetics and reversibility of monomer binding is the same at \( 37^\circ \text{C} \) (27). This finding seems appropriate since it would be impossible for macrophages to maintain endocytosis and degradation of the monomeric IgG normally found in serum. However, immune complexes of dimeric IgG or larger, once bound to the macrophage membrane, are rapidly endocytosed and catabolized (28, 29). More importantly, the endocytic rate does not increase with an increase in complex size (28, 29). Thus only two Fc receptors need to interact for irreversible endocytosis to occur. An important assumption here is that the IgG FcR has a valency of a single Fc unit and therefore binds a single immunoglobulin. This assumption is based on the fact that the IgE FcR has such a valency (30, 31, 32).

1.4C Biochemical Studies

Young et al. have studied the effects of specific ligand binding, to the IgG FcR, on the membrane potential of a macrophage cell line. Rapid membrane depolarization was observed within 20 seconds of ligand binding. The extent of membrane depolarization was found to be dependent on the cross-linking of receptors or the
cross-linking of ligand bound to those receptors. The depolarization was also determined to be due to Na⁺ influx into the cell. This determination was made by eliminating Na⁺ from the cell incubation media and again observing the membrane potential change (Ψ). In this same manner extracellular Ca²⁺ was found not to play a significant role. These same investigators also studied the effect on membrane potential changes of three other ligands which recognize other major membrane surface antigens on the same monocyte line. A similar Ψ was not observed. This phenomena appears to be specific for the FcR. From these studies Young has suggested that the FcR is a ligand-dependent ion channel and that the Ψ that occurs activates subsequent cellular responses [33].

1.4D IgG FcR Structural Investigations

It is strongly believed that the IgG FcR has a subunit structure although there have been no findings to support the hypothesis thus far. The investigations of Young and Unkeless [4] actually suggest that the FcR functions independently. Young and Unkeless purified the IgG FcR from a monocyte cell line using a monoclonal antibody. The FcR material was then reconstituted into proteoliposomes and planar bilayer membranes. In the presence of specific ligands the purified receptor increased the cation permeability of the proteoliposomes. The FcR was thus capable of initiating a response without the presence of any other associated proteins.

1.5 CROSS LINKING REAGENTS

Chemical cross-linking reagents are short linear molecules that contain reactive groups at both ends. The reactive groups may be specifically directed toward a particular class of functional groups on macromolecules, or may have no specificity at all. The primary use of these compounds is in structural studies, particularly on cell membranes. Membrane molecules that are within close proximity of each other become "bridged"
by these reagents and are then analyzed. The subunit stoichiometry of several protein systems have been determined in this manner (35, 36).

The use of cross-linking reagents is particularly popular for structural investigations in the 5-20 Angstrom (Å) range. At 5 Å or less, various types of spectroscopy and diffraction procedures provide definitive information. Electron microscopy is useful above 20-30 Å. The information obtained from cross-linking studies is not as precise as the physical techniques yet has been very valuable. Quaternary structure of oligomeric proteins and even larger molecular aggregates can be unambiguously established. A comparison of results obtained from cross-linking experiments and crystallography on proteins of known structure found the two methods to be in agreement.

The utilization of cross-linking reagents in structural studies of biological systems provides other advantages. The information is obtained from such studies without altering the biological function of the molecule. In addition, straightforward results are achieved since these reagents do not contribute to molecular weight.

The cross-linking reagents are not without their disadvantages. First of all, it is often difficult to establish optimal conditions for cross-linking reactions. Also, in protein solutions that are sufficiently concentrated, there is increased formation of cross-linked collision complexes which can provide misleading information. Another problem arises when many different macromolecules are present in the system. Analysis of such mixtures can be very difficult. Finally, when cross-linking intact cells, the reagents can be so effective that the cells cannot be lysed for subsequent analysis.

1.5B Cross-Linking Reagent Categories

There are many different cross-linking reagents available commercially or which can
readily be synthesized. General categories of these reagents include homobifunctional, heterobifunctional and photosensitive. The homobifunctional class of reagents contains the same reactive group at each end while the heterobifunctional contains two dissimilar groups. The photosensitive reagents can be either homobifunctional or heterobifunctional and contain reactive groups (one or both) which cross-link only upon exposure to light.

These three types of reagents are available in various lengths and cleavable forms. The shorter the reagent, especially at 5 Å or less, the fewer the cross-links formed. Cleavable cross-linking analogs contain either disulfide or glycol bridges within their structure. The disulfide linkages can be cleaved by reducing agents such as 2-mercaptoethanol [2-ME] while the glycol bridges are cleaved by sodium periodate. The cleavable cross-linkers are especially invaluable in structural studies. Individual components of a cross-linked complex can be identified following cleavage of the cross-linking compound.

1.5C Photosensitive Cross-linking Reagents

When appropriate functional groups of a specific macromolecule are not available for cross-linking, reagents of high reactivity and low specificity can be used. The photochemical cross-linking reagents are one such group which is gaining popularity. These reagents remain totally inert until a photon of light is absorbed. Upon light absorption an intermediate reactive species is generated which can then become attached onto biological molecules. The concentration of the reactive species formed will depend directly on the number of light quanta absorbed and indirectly on the concentration of the precursor. Shortwave ultraviolet lamps are most commonly used to provide the necessary light for photolysis. If flash photolysis is desired, ordinary camera flash units can be used. For this particular investigation a xenon lamp, which could be pulsed, was utilized.
The photosensitive reagents have gained increasing popularity since they overcome inherent problems of conventional reagents such as random collision cross-links, long reaction times and difficulty in controlling reactions. Photolysis in the millisecond range can initiate cross-linking reactions that provide a picture image of interacting systems at the desired moment. Random collision cross-links still occur, but at a much lower frequency. By cross-linking with heterobifunctional photosensitive reagents, cross-linking is not only rapid but can also be controlled sequentially. Cross-linking with these reagents involves two steps: the first step being a dark incubation for the reaction of the conventional end. The photoreactive precursor can then be manipulated into a desired position prior to photolysis.

Reagents that have proven useful for photolabelling/photochemical cross-linking include precursors to carbenes, nitrenes and free radicals (37). Discussion will be limited to the nitrenes.

Nitrenes are generated photochemically from suitable aryl or alkyl azides. The general reaction mechanism of nitrene formation from an aryl azide is shown in Fig. 8. The half life of the intermediate reactive species is of the order of $10^{-4}$ to $10^{-2}$ seconds. Once a nitrene is generated it may undergo any of the following reactions: 1) hydrogen abstraction 2) insertion at C-H bonds 3) addition 4) condensation 5) insertion at N-H bonds and 6) rearrangement (66). These reactions are illustrated in Figure 9.

The most widely used class of photolabel precursors is the aryl azides. Their present popularity stems from the ease with which they can be synthesized, their stability in storage and their lack of reactivity under physiological conditions in the absence of light. In addition, the aryl nitrenes are less susceptible to internal rearrangement than are the alkyl nitrenes. Furthermore, the absorption maxima for the alkyl nitrenes is 290 nm: a wavelength at which damage can occur to proteins and nucleic acid systems.
Figure 8. General reaction mechanisms of a) imidates and b) N-hydroxysuccinimide esters with primary amino groups. c) Illustrates nitrene generation from aryl azides.

a) AMIDINE BOND FORMATION

\[
\begin{align*}
\text{NH}_2^+ & \quad \text{R-C-O-CH}_3 + \text{NH}_2-P \\ \text{R-C-NH-P} & \quad + \text{CH}_3\text{OH}
\end{align*}
\]

b) AMIDE BOND FORMATION

\[
\begin{align*}
\text{R-C-O-N} & \quad + \text{NH}_2-P \\ \text{R-C-NH-P} & \quad + \text{HO-N}
\end{align*}
\]

N-HYDROXY-SUCCINIMIDE ESTER

c) NITRENE FORMATION

\[
\begin{align*}
\text{N=N=N} & \quad \text{R-NO}_2 \quad \rightarrow \quad \text{-N}_2 \quad h \nu \\ \text{R} & \quad \text{R-NO}_2
\end{align*}
\]

ARYL AZIDE
Figure 9. Some possible fates of nitrenes

1) \( \text{R-N} + \text{H-C} \rightarrow \text{R-NH} + \cdot \text{C} \rightarrow \text{other products} \)
2) \( \text{R-N} + \text{H-C} \rightarrow \text{R-NH-C} \)
3) \( \text{R-N} + \text{H-C} \rightarrow \text{R-N} \)
4) \( \text{R-N} + \text{R-N} \rightarrow \text{R-N=N-R} \)
5) \( \text{R-N} + [\text{R'}_2] \text{NH} \rightarrow \text{R-NH-N}^{\text{R'}} \)
6) \( \text{R-N} \rightarrow \text{rearrangement products} \)

1) hydrogen abstraction, 2) insertion at C-H, 3) addition, 4) condensation, 5) insertion at N-H, and 6) rearrangement.
Substituted aryl azides have been developed that can be photolyzed in the near visible light region; wavelengths at which such protein damage cannot occur.

1.5D Experimental Design

The use of cross-linking reagents is not technically difficult, however the challenge lies in finding optimal conditions for the reagent within the restrictions of the experimental system. Attention must be given to the reaction time period, temperature, pH, buffer composition and cross-linker concentration. These factors will vary with the reagent in use and conditions for the more widely used reagents have been well established. Of note here is reagent concentration since many of these compounds are extremely unstable and will undergo hydrolysis upon solubilization. For this reason reagent excess is required for quantitative cross-linking to occur. To avoid adverse side reactions several successive additions of reagent is preferred.

When performing cross-linking on cell membrane proteins, further consideration must be given to experimental design. From the Fluid Mosaic Model [8], membrane proteins can be peripheral or integral. Integral proteins, as are receptor molecules, are strongly associated with the membrane phospholipid. These proteins can either face the cell cytoplasm or the exterior of the cell, or can be transmembraneous, and span the entire membrane. For those proteins that face inward, suitable reactive groups may be difficult to reach with cross-linking reagents. For this reason cross-linking of cell lysate material should also be performed.

1.5E Cross-linking Reagents Utilized

Imidates are the most common cross-linking reagents used for cell membrane structural studies. Imidates are imido esters and have the general formula $R-C'\text{O}_2\text{OR}$ \text{NH}. Those imidates that are symmetrical are referred to as bisimidates. Imidates are water
soluble and most are permeable to cell membranes. Depending on the reagent's rate of hydrolysis, half-lives range from several minutes to 30 minutes. Because this hydrolysis occurs, up to 100 fold reagent excess may be required. For a more complete review of these compounds the investigator is referred to Peters and Richards [36].

The primary reaction of imidates involves formation of an amidine bond with primary amino groups on protein; a reaction referred to as amidination. The actual mechanism of amidination has been elucidated by Hand and Jencks [39] and is illustrated in Figure 8.

Temperature and pH significantly effect the imidate reaction. For maximum formation of amidine bonds with a minimum of side products, the reaction should be carried out at pH 10 or slightly above [40]. In many cases this is not feasible since proteins, and in particular cells, do not tolerate such harsh alkaline conditions. On the other hand, if the pH of the reaction system is at a pH of 8 or lower, side reactions are more predominate. As regards temperature, the reaction rate decreases severalfold as the temperature is lowered from 39°C to 25°C, and then again from 25°C to 0°C. From previous cross-linking experiments, optimal conditions for reaction with imidates are pH 9.3, 30-60 minute incubation at room temperature and a total reagent concentration of 1-5 mM with incremental additions every 10-30 minutes [41,42]. These conditions are idealistic and serve only as a guideline as actual conditions should be optimized for the particular experimental system under consideration.

As mentioned earlier, DMS and DTBP were to be the two imidates utilized. Both of these reagents are homobifunctional. DTBP represents the cleavable analog of DMS as it contains a disulfide bridge in its structure. The molecular weights of DMS and DTBP are 273 and 281 respectively. The chain length of DMS is 11 Å and that of DTBP is 11.9 Å. Their structures can be seen in Figure 4.

Cross-linking attempts were also to be made with an N-hydroxysuccinimide ester in
Figure 4. Structures of the cross-linking reagents. (a) and (b) show homobifunctional bisimidates. (a) represents cleavable dimethyl-3, 3'-dithiobispropionimidate (DTBP) and (b) dimethyl suberimidate (DMS). (c) represents the heterobifunctional photosensitive N-5-azido-2-nitrobenzoyloxsuccinimide (ANB-NOS) with its n-hydroxsuccinimide ester end and (d) disuccinimidyl suberate (OSS) also containing such a group.
order to evaluate whether different cross-linked products could be formed using reagents with different reactive groups. These reagents are known to react primarily with amino groups to form an amide bond (43) as can be seen in Figure 8. These reagents are unstable in aqueous media and have a half life on the order of 10 minutes at pH 8.6 and 4°C (44) and several hours at pH 7.0 and 0°C (45). Reactions are complete within 10 minutes at 0-4°C (44,45). DSS was to be the N-hydroxysuccinimide ester in use and its structure can be seen in Figure 4. DSS has a molecular weight of 368 and a chain length of 12 Å. Since DSS is more highly reactive, extensive cross-linking can occur so that much lower reagent concentrations must be used.

The photosensitive reagent in use, ANB-NOS, contains an N-hydroxysuccinimide ester as its conventional group and a substituted aryl azide as its photosensitive group. ANB-NOS is called a substituted aryl azide since it contains a nitro group on its aromatic ring. This substitution enhances the reactivity of the photogenerated aryl nitrene and allows photoactivation at 320-350 nm. ANB-NOS has a molecular weight of 305 and its structure can also be seen in Figure 4. The distance between the epsilon amino and azido group, upon protein modification, is 6.5 Å (46). Because of the nonspecific reactions of nitrenes, an interpretation of chain length and optimal cross-linking is still rather difficult at this time.

1.6 U937 CELLS

The cells for this study were a human monocyte line designated U937. U937 is a tumor cell line derived from a patient with histiocytic lymphoma. These cells are more easily maintained in large numbers and pure form, than are normal monocytes. Cell numbers can be doubled in 24 hours and densities of 1.5 x 10⁶ cells/ml are possible (Anderson unpublished results). In addition to providing a ready supply of cells, the U937 FcR should provide a reliable model for the FcR structure and function in normal human monocytes. Binding and kinetic studies performed on the U937 IgG FcR using 16
radiolabelled human myeloma proteins have shown strong similarities to normal monocytes [27]. As with the normal monocytes, IgG1 and IgG3 were found to bind more efficiently to the U937 FcR, while IgG2 and IgG4 bound less readily. U937 cells also share such features with normal macrophages as strong esterase activity, lysozyme and endogenous pyrogen production, phagocytic capabilities and complement receptors [47,48]. U937 cells are lacking in such characteristics as myeloid colony-stimulating activity [49], lymphocyte activating factor [50] and prostaglandin E [49]. Kurland et al. suggest that these missing features indicate a lack of differentiation [50].

Purified normal human monocytes were to be utilized in key procedures so that a true picture of normal endocytosis could be obtained. These cells would have been obtained from peripheral blood using a counter flow centrifugation-elutriation technique [51].

1.7 PRELIMINARY STUDIES

1.7A IgG FcR Characterization

The IgG FcR on U937 cells and normal peripheral monocytes has been identified as a 72,000 dalton glycoprotein using classical affinity column chromatography methodology [52]. These investigations also revealed a possible second minor component of 40,000 daltons. In brief, detergent lysates of chloroglycouril iodinated U937 cells were made in 1% NP-40. The lysates were then passed through a Sepharose-IgG affinity column and the column eluted with acetic acid. The acid eluates were then analyzed by SDS-PAGE and autoradiography.

Anderson was able to show that the p72 molecule, isolated from the above procedure, is all or part of the IgG FcR on the basis of the following findings: a) radiolabelling of the molecule using chloroglycouril was blocked by occupation of the FcR with IgG b) in soluble form this molecule expressed ligand specificity identical to the in situ
receptor c) the molecule was not recovered from affinity adsorbents bearing proteins that do not bind to the Fc receptors, nor d) from a human T cell line that does not bear Fc receptors [52].

The significance of the p40 molecule has not been fully clarified. Actin appears to make up at least part of this band since the p40 was found to migrate comparably with the actin marker on two-dimensional electrophoresis. The remaining portion of the p40 band seems to have a molecular weight that is slightly higher and has isoelectric points much more basic than actin [52]. Since actin is a contractile protein of the cell membrane cytoskeleton, one can speculate that the p40 may be a transducer domain of the FcR.

1.7B Cross-linking Studies

The ability to achieve cross-linking with DMS, of molecules situated on the surface of U937 cells, has also been tested. U937 cells were incubated with radiolabelled IgG to allow binding of the radioligand to the cell prior to cross-linking. As a matter of comparison, samples that were not to be cross-linked received comparable amounts of the DMS solvent. The cross-linking reaction was quenched with glycine and the cells then lysed. Lysed material was then prepared in Laemmli's Sample Buffer containing 2-ME for the SDS-PAGE procedure. Following processing of the gel, a prefogged autoradiographic film was apposed to it. The autoradiogram (AR) was allowed to develop for a length of time which varied with the amount of radioactivity loaded on to the gel. The developed AR revealed radioactive bands at molecular weights corresponding to various combinations of linked immunoglobulin heavy chain, light chain and receptor when DMS was present (Anderson unpublished results). When DMS was not present bands were visualized at molecular weights of 50,000 and 22,000 daltons only, the approximate molecular weights of immunoglobulin heavy chain and light chain respectively.
Once it was ascertained that cross-linking could be achieved with DMS, Anderson then attempted to cross-link the U937 FcR p72 molecule to a neighboring molecule while the cells were intact. The protocol used was similar to the aforementioned procedure except that after cell lysis the FcR was immunoprecipitated from the lysates using either goat anti-FcR|| (a goat anti-FcR antibody developed by Anderson) or aggregated IgG (AgglgG). Heat fixed Staphylococcus aureus Cowan I (SAC I) was then added to all samples. This particular Staph strain contains a membrane protein called Protein A which is capable of binding the Fc portion of an IgG. These SAC "beads" add weight to the entire immune complex so that they can be removed by centrifugation. (With regards the AgglgG, one assumes that not all Fc regions are occupied with receptor.) The radioactive material attached to the SAC beads was then separated by SDS-PAGE and visualized by AR. The results are reflected in the Table below. Besides the FcR being present, two new molecules were immunoprecipitated with the goat anti-FcR||, a 170,000 and a 350,000 dalton protein. AgglgG did not precipitate these bands. The p170 molecule was present with or without the presence of DMS and has been attributed to nonspecificity of the goat anti-FcR||. Thus, the only product of interest is the molecule having an approximate molecular weight of 350,000 daltons. The individual components of the p350 were to be the subject of analysis undertaken within this project.

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Table 2. AR results of DMS cross-linked intact U937 cells. The numbers indicate molecular weights in daltons.
1.8 PROPOSED INVESTIGATIONS

The primary objective of this study is to identify a near-neighbor molecule to the FcR on human macrophages with the consideration that the neighboring molecule may also be involved in FcR mediated endocytosis. The investigations will be performed on a cell line designated U937.

FcR near neighbor analysis will be attempted using several cross-linking reagents. The two midates, DMS and DTBP will be the primary reagents utilized. Depending on the success of these reagents, other cross-linking reagents with different reactive groups and chain lengths will also be considered.

Preliminary cross-linking attempts by Anderson using DMS on intact U937 cells have yielded a cross-linked product with a molecular weight of 350,000 daltons. My experimentation will begin with attempting to reproduce these same results. If it is ascertained that the p350 molecule can be reproduced, a second cross-linking experiment will be performed using DTBP. Two-dimensional gel electrophoresis following DTBP cross-linking should then characterize the molecular weights of the individual components of the p350 molecule.

Cross-linking of Fc fragments of IgG and Fab fragments at anti-FcR|| to the radiolabelled U937 cell FcR will be attempted. By using specific Fc and Fab fragments of known molecular weights (approximately 50,000 daltons), it can be ascertained whether close lying molecules are capable of being cross-linked to the FcR. The efficacy of cross-linking can also be analyzed in this manner.

Depending on the time frame, cross-linking will be attempted on plasma membrane vesicles. These vesicles are formed by homogenizing cells in the absence of detergent. Vesicles formed can be inside or right side out. Inside out vesicles will allow access to FcR near neighbors on the cytoplasmic portion of the membrane.
20

SECTION II

MATERIALS AND METHODS

2.1 U937 Cells

**Cells.** Cultures of U937 cells were obtained from Dr. Peter Ralph, Sloan-Kettering Institute for Cancer Research, Rye, NY. These cells were maintained in spinner culture in RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum (FCS) (Sterile Systems, Logan, UT), penicillin (100U/ml), and streptomycin (100 g/ml), both from Gibco Laboratories.

2.2 Iodination

The U937 cells were radioiodinated by the chloroglycouril method (64). 0.7 ml of cells (14.3 x 10^6 cells/ml of PBS) and 1 mCi ¹²⁵I (IMS.300, Amersham, Arlington Heights, IL) in 10 µl were placed in a vial coated with 5 µg chloroglycouril and incubated for 30 minutes at 0°C. The reaction was quenched and the cells were washed three times in 5 mM KI in PBS. The cells were then either resuspended in bicine pH 9.5, a Good Buffer (55) to 3.0 x 10^6 c/ml, or lysed in 1% Nonidet P-40 (NP-40). 2 mM phenylmethylsulfonylfluoride (PMSF), and aprotinin (1 Trypsin inhibitor unit [TIU/ml]) in PBS for 30 minutes at 0°C, depending on whether lysate or whole cells were used for cross-linking. The cell nuclei and unlysed material were sedimented at 10,000g for 30 minutes at 4°C.

2.3 SDS-PAGE and Autoradiography

SDS-PAGE was performed on gradient slab gels according to the method of Laemmli
The acid eluate samples containing 2% SDS, 10% glycerol, 5% 2-ME, and 0.001% bromphenol blue, were immersed in boiling water for 2 minutes and applied to the stacking gels. Molecular weight markers were myosin (200,000), B-galactosidase (130,000), phosphorylase B (94,000), Bovine Serum Albumin (BSA) (68,000), ovalbumin (OA) (43,000), soybean trypsin inhibitor (21,000), and lysozyme (14,300), from Bio-Rad Laboratories and chloramine T-radioiodinated human IgG1 (27). After being stained, destained and dried, the gels were apposed to pre-fogged Xomat AR X-ray film (Eastman Kodak Co., Rochester, NY) and placed in Kodak film cassettes at -70°C. Two dimensional gel electrophoresis was performed on 5 - 10% gradient slab gels in both dimensions. Sample buffer was the same as that noted previously except that 2-ME was not present in the buffer used in the first dimension. The lane of interest was outlined using fluorescent markers, dansylated BSA and dansylated ovalbumin (57), (dansyl chloride from Bio-Rad Laboratories) in adjacent lanes. The markers were visualized with a UV lamp while the lane was cut out. The entire lane was then soaked in LSB+ for a minimum of 2 hours. Electrophoresis was performed in the second dimension by placing the lane at the top of a second gradient slab gel and anchoring it with agarose (Marine Colloids Inc., Rockland, ME).

2.4 Reagents

The following reagents were obtained from Sigma Chemical Co. (St. Louis, MO): BSA, OA, cytochrome c, aprotinin, PMSF, TRIS, L-methionine, glycine, bicine, tricine, DMSO, NP-40 and trichloracetic acid (TCA). EDTA, potassium iodide (KI), sodium carbonate (Na₂CO₃), and ammonium persulfate were obtained from Fisher Scientific (Fair Lawn, NJ). Sodium azide (NaN₃), bromphenol blue and acetone was obtained from Eastman Kodak. Sodium chloride (NaCl), sodium hydroxide (NaOH), and dioxane were obtained from Mallinckrodt (St. Louis, MO). Sodium dodecyl sulfate (SOS), N, N' methylene-bis-acrylamide, acrylamide and temed were obtained from Bio-Rad Laboratories. IgG Sorb was obtained from the Enzyme Center (Malden, MA). Pooled
human IgG was purchased as Pentex human gamma globulin and further purified by DEAE cellulose ion exchange chromatography. A human IgG1 myeloma protein (Woo) and Fc fragments from the same protein, as well as a human IgG myeloma (Allendorf) and Fab fragments of goat anti-FcR|1 were purified as described (27). Aggregated IgG (Agg IgG) was prepared by heating human IgG at 10 mg/ml to 63°C for 12 minutes. The medium in which U937 cells were incubated with IgG1 to saturate Fc receptors was balanced salt solution (BSS) (58) containing BSA (1 mg/ml) and NaN3 (0.2%) (BBA).

2.5 Cross-linking of intact U937 Cells

Dimethylsuberimidate (DMS), dimethyl-3.3'-dithiobispropionimidate (DTBP) and disuccinimidyl suberate (DSS) were all purchased from Pierce Chemical Company (Rockford, IL). N-5-azido-2-nitrobenzoyloxsuccinimide (ANB-NOS) was synthesized by Sheldon Isaacson in the summer of 1980 in Dr. Clark Anderson's laboratory, University of Rochester (Rochester, NY). The ANB-NOS was stored refrigerated in a brown bottle covered with aluminum foil. All cross-linking reagents were prepared fresh just prior to addition and all reactions were performed on ice. Cross-linking was performed on 2.5 ml aliquots of radiolabelled U937 cells resuspended to 3.0 x 10^6 cells/ml in the buffer indicated for the particular cross-linker.

Surface-labelled U937 cells were chemically cross-linked with DMS after resuspension in a bicine pH 9.5 buffer. Bicine buffer pH 9.5 was used to maintain a pH of 8.5 during cross-linking (20). DMS was prepared at 90 mg/ml of 0.1 M Na2CO3. DMS was added to (3) 2.5 ml aliquots of the cell suspension; the initial concentrations of the DMS being 1, 3 and 6 mM. After the second addition, final DMS concentrations were 3, 6 and 10 mM. Incubation after the first DMS addition was for 1 hr, mixing every 15 minutes. The second incubation was for three hours with mixing. Subsequent experiments revealed sufficient cross-linking with 2 incubations of 30 minutes. Cross-linking was then quenched with 0.5 M glycine in PBS (at least 20 M excess needed).
5 mM KI/50 mM glycine in PBS was used to wash the cells two times prior to lysis. The cells were lysed at 50 x 10^6 cells/ml as described in 2.2. FcR immunoprecipitation then followed.

A positive control consisted of cross-linking IgG-Woo coated U937 cells with DMS after two additions at 0 and then 10 mM. For the negative control, bicine buffer pH 9.5 was added in comparable amounts to the cross-linking reagent. The cells were then lysed with 1% NP-40/PMSF/TRAS* (see note below) and the TCA or acetone precipitates of the lysates were analyzed by SDS-PAGE.

DTBP was prepared and used in the exact same manner as DMS.

Washed 125I labelled U937 cells were resuspended in PBS prior to cross-linking with DSS. DSS was prepared at 50 mM in DMSO. Two DSS additions were made for concentration ranges of 0.5 to 1.0, 1.0 to 2.0, and 2.0 to 4.0 mM. DSS addition was followed by 10 minute incubations. Quenching and further processing was also the same as for DMS.

Labelled U937 cells were prepared in PBS for cross-linking with ANB-NOS. ANB-NOS was prepared at 100 mM in dioxane and protected from light at all times. Dioxane was kept at 10% of the protein. Final ANB-NOS concentrations achieved for the single 15 minute "dark" incubation were 1, 5, 10 and 20 mM. Higher concentrations of ANB-NOS resulted in a precipitate due to excessive derivitization of cellular proteins by the NOS end. All sample tubes were foil covered and capped. For comparison, one sample with 20 mM ANB-NOS was kept in the dark for the entire procedure. A negative tube with dioxane addition alone was also run. Following the dark incubation, the samples were photolyzed with a xenon lamp at 6000 volts, 14 pulses/second for 5 minutes. Quenching and further processing was then as before.

*Trasylol is abbreviated TRAS and is equivalent to aprotinin.
2.6 Cross-linking of Lysed U937 cells

$^{125}$I labelled U937 cells were washed in 5 mM KI/PBS twice, once with 5 mM KI/bicine pH 9.5, then solubilized with 1% NP-40 in bicine pH 9.5. No protease inhibitors were added during solubilization so all procedures were maintained at 4°C and performed without delay. High speed centrifugation of the extracts was as before. 100 $\mu$L of the lysates were cross-linked by two additions of DMS in the following range: 1 to 2, 2 to 4, 3 to 6, 4 to 8 and 6 to 12 mM. The latter concentration represents the final DMS concentration. Incubations were for 30 minutes following each addition. 1% NP-40 in PBS was used to prepare cell extracts for DSS cross-linking. 100 $\mu$L of lysate was used and DSS concentrations and incubation times were the same as for intact cells. Quenching was again with 0.5 M glycine/PBS. Sample volumes were kept as small as possible for FcR immunoprecipitation.

2.7 FcR precipitation

The FcR was immunoprecipitated from lysed radiolabelled U937 cells in two ways: using goat antibody to the FcR ($\geq$FcR$\|\$) at 13.9 mg/ml, and aggregated IgG (AgglG) at 10 mg/ml. To one tube of 50 $\mu$L of lysate was added 2 $\mu$L of AgglG and to a second tube was added 7 $\mu$L of $\geq$FcR$\|\$. After 30 minutes 200 $\mu$L of a 10% suspension of Staphylococcus aureus Cowan I (IgGSorb or SAC bacteria) was then added to all samples to adsorb IgG immune complexes. After another 30 minutes the samples were centrifuged at 3000 rpm for 15 minutes at 4°C to yield a firmly packed pellet of immune complexes. A series of washes using SAC buffers \cite{60} was then performed. After the final wash, 80 $\mu$L of Laemmli's Sample Buffer was added to the pellets to dissociate the immune complexes upon boiling, in preparation for SDS-Page. All samples were counted on a gamma counter prior to loading to the gels to determine the time exposure for autoradiography.
2.8 Cross-linking of fragment coated U937 cells.

$^{125}\text{I}$ labelled U937 cells were washed in BBA once and resuspended in the same to 30 x 10$^6$ cells/ml. (3) 0.5 ml cell samples were prepared for separate incubations with IgG$_1$-Woo Fc fragments at 18.7 mg/ml, $>\text{FcR}_{\text{I}}$ Fab fragments at 9.34 mg/ml and with BBA alone. 54 ml of each were added to the cell suspensions and binding was allowed to occur for 1 hour at 4°C. Following this incubation the cells were washed once in BBA, twice in BSS and resuspended in PBS to 3 x 10$^6$ cells/ml for cross-linking. 2.5 mls of each suspension were reacted with DSS at initial concentrations of 0.5 and 1 mM and with second additions of DSS for final concentrations of 1 and 2 mM. Incubation times after each addition were 10 minutes on ice. Quenching, cell solubilization, immunoprecipitation and analysis was as before.

2.9 TCA precipitation

TCA precipitations performed on lysate material were used to monitor the recovery of radioactivity and success of the labelling procedure. 10 ml of cell lysate was used to make a 1:100 dilution in 1% NP-40. 10 ml of the diluted sample was then added to 0.5 ml BSA/PBS at 5 mg/ml and 0.5 ml of 25% TCA and incubated on ice for 20 minutes. The sample was spun at 3000 rpm for 10 minutes at 4°C. The supernatant was carefully removed and saved. Counts were taken on the supernatant and pellet.
Section III

RESULTS

3.1 Cross-linking of intact U937 cells

3.1A DMS Utilization

When $^{125}$I-surface labelled U937 cells are cross-linked with DMS for 4 hours and solubilized with 1% NP-40/PMSF/TRAS, goat anti-FcR\textsubscript{II} immunoprecipitation yields a slight band with a molecular weight of 72,000 and denser bands at 170,000 and 350,000 on autoradiogram (AR). When no DMS is present bands can be seen at 170,000 only. Immunoprecipitation with AgglG did not yield any bands. These last two observations suggest that a loss of FcR occurred during the procedure. The positive control, which consisted of cross-linking U937 cells coated with *IgG-Woo, indicated that cross-linking was successful since radioactive bands were seen at molecular weights which correspond to combinations of heavy and light chains.

3.1B Analysis of FcR loss

Before any further cross-linking studies were done an analysis of pH effects, bicine toxicity and long cross-linking incubations on FcR recovery was performed. This analysis was performed in the following manner. Surface radiolabelled U937 cells were resuspended in 5 mM KI in PBS, divided into four aliquots and centrifuged at 930 rpm, 4°C for 7 minutes. The cells of the first aliquot were lysed as before. The other three aliquots were resuspended to 3 x 10$^6$ cells/ml in BSS, bicine pH 7.4 and bicine pH 8.5 and put on ice for 4 hours with mixing every 15 minutes. Lysis of the three cell samples was then performed with 1% NP-40/PMSF/TRAS followed by FcR immunoprecipitation and SDS-PAGE. TCA precipitations were performed on the lysates to determine
recovery. Labelled U937 cells maintained in bicine pH 7.4 or BSS yielded bands on AR after FcR immunoprecipitation that were somewhat more dense than those cells maintained in bicine pH 8.5. These results suggest that pH may have a small effect on FcR recovery and that bicine is only minimally toxic to the FcR. However, the cells in BSS that were not processed through an incubation period, yielded bands significantly denser than the other samples above. Thus it was concluded that the FcR was not clearly visualized following DMS cross-linking due to loss of radioactivity. Incubation times of 30 minutes were used for subsequent procedures.

3.1C DTBP Utilization

Cross-linking with the cleavable analog DTBP for 30 minutes yielded results that were comparable to those from the preliminary studies done by Anderson when DMS was used for 30 minutes. Anti-FcR\| precipitation yielded bands on AR at 72,000, 170,000 and 350,000. A single disperse band centered at 72,000 as well as dense bands stacked at the interface of the stacking and running gel were seen with Agg\lgG as the precipitating agent. (See Figure 5) The dense bands at the top of the gel probably represent larger cross-linked products incapable of entering the gel.

In order to identify the individual components of the 350,000 cross-linked molecules achieved with DMS and DTBP, two-dimensional gel electrophoresis was performed as described in Methods following cross-linking with DTBP in the 6 to 10 mM range. Of the original bands, the 350,000 molecule alone was cleaved into smaller products as seen by an AR of the 2-D gel. (Figure 6) The cleaved product had a molecular weight of 170,000 indicating that the p350 is a dimer of the original p170 and thus is unrelated to the FcR. The 350,000 band did not entirely disappear after reduction indicating that there was incomplete DTBP cleavage or that there may be impurities in the cross-linking reagent. Also of interest on the 2-D gel AR was the appearance of the p72 molecule. Although it still migrated as a disperse band in the molecular weight region between 60,000 and 80,000, it appeared heterogenous as several distinct spots could be distinguished.
Figure 5. Autoradiogram of SDS-PAGE gradient slab gel (5-10%) analyzing the immunoprecipitation products following DTBP cross-linking of intact U937 cells. The cells were lysed in 1% NP-10/PMSF/TRAS prior to SDS-PAGE. SDS-PAGE was performed under nonreduced conditions. Products in Lanes 3 through 6 are from AgglG immunoprecipitation while anti-FcR∥ was used in the samples in Lanes 8 through 11. DTBP was highest in Lanes 5 and 10. Samples from Lanes 6 and 11 received no DTBP. Bands can be seen stacked at the top of the gel. Numbers to the right of the autoradiograph indicate apparent molecular weight x 10⁻³. T = top of 5 - 10% gel. D = bromphenyl due front.
Figure 6. Autoradiogram of the SDS-PAGE two-dimensional gel analyzing the p350 cross-linked molecule produced from DTBP cross-linking of intact surface-labelled U937 and immunoprecipitated with anti-FcR\(_\perp\). The second dimension was run in the presence of 2 ME. Due to incomplete cleavage some of the p350 remained (spot 1). Spot 2 is a cleavage product of the p350 and has a molecular weight of 170. Spots 3 and 4 did not cleave and represent the original p170 and p72 (FcR) molecules respectively. Note the very disperse FcR band.
Figure 7. Autoradiograms of SDS-PAGE gradient slab gels (5-10%) analyzing the immunoprecipitation products following cross-linking of soluble extracts of surface labelled U937 cells using (A) DMS and (B) DSS. In (A) Lanes 1 through 6 are products of anti-FcR\textsubscript{II} precipitation. DMS concentrations decrease from Lanes 1 to 5 and 8 to 12. Lanes 6 and 13 contained no DMS. In (B) Lane 1 is missing. AgglG was used for Lanes 2 to 5 and anti-FcR\textsubscript{II} was used for Lanes 7 to 10. Lane 11 is a radiolabelled IgG-Woo marker. DSS was also used in decreasing concentrations from Lane 2 to 4 and 7 to 9. No DSS was used in samples in Lane 5 and 10. Numbers to right of autoradiographs indicate apparent molecular weight $\times 10^{-3}$. T = top of 5-10% gradient gel. D = bromphenyl blue dye front. * indicates slight band at 140 in Figure A.
3.1D ANB-NOS cross-linking

A U937 cell suspension at $40 \times 10^6$ cells/ml of BBA was incubated with $^{125}\text{I}$ labelled IgG-Allendorf to allow binding to occur as was done for the control cells from previous procedures. Following cross-linking of the intact U937 cells with ANB-NDS the AR showed bands at the following molecular weights: 22,000 (light chain), 50,000 (heavy chain), 95-100,000 (2 heavy chains) and 140,000 (an intact immunoglobulin IgG). No bands were seen that would indicate cross-linking to the FcR had occurred.

3.2 Cross-linking of $^{125}\text{I}$ lysed U937 cells

$^{125}\text{I}$-surface labelled U937 cells were solubilized in 1% NP-40/bicine pH 9.5, without the presence of enzyme inhibitors for cross-linking with DMS. AgglG immunoprecipitation of the soluble extract following cross-linking yielded a very slight band on the autoradiogram at 140,000 when DMS was at its highest concentration range of 6 to 12 mM. The usual disperse band at 72,000 was also seen. This was the first time that AgglG yielded anything other than the p72. Immunoprecipitation with anti-FcR\| yielded a disperse 72,000 dalton band as well as bands at 130,000, 170,000 and 350,000. The 130,000 band was also very slight. (See Figure 7) The gel was put back to film for a two week period of time so that the p140 from AgglG precipitation could be visualized more clearly. The two week AR did show slight bands at 140,000 in all lanes precipitated with AgglG when DMS was present while this p140 band was not present in the absence of DMS. These bands were so slight that it would have been difficult to analyze the molecule any further.

Cross-linking of soluble extracts of U937 cells with DSS yielded no bands on AR that had not been seen with the other reagents. A p350 cross-linked product was also achieved with DSS. Denser bands were seen with decreasing DSS concentrations suggesting that over cross-linking may have been a problem despite the low concentration of DSS used. (See Figure 7) At higher DSS concentrations the cells can
not be lysed properly such that the FcR is recovered in smaller amounts or not at all. Centrifugation following lysis of the cells did indeed yield NP-40 pellets larger than normal and which did not have the usual stringy appearance.

3.3 Cross-linking of fragment coated U937 cells

Fab fragments of goat anti-FcR\textsubscript{II} and Fc fragments of IgG-Woo were coated onto separate samples of radiolabelled U937 cells and cross-linking then attempted on the cells with DSS as described in Methods. Incomplete lysis of the cells again posed a problem. The bands seen on AR were similar to those from all the other cross-linking attempts.
With regards to the objective of this research, a neighboring molecule to the FcR has not been identified. It cannot be concluded however, that the FcR does not have molecular associations with neighboring molecules or that the FcR does not have a subunit structure. It is clear that the conditions used were adequate for cross-linking with the reagents used since the control cells coated with radiolabelled IgG and cross-linked gave positive results and $170 + 170 \approx 350$. Thus a suitable conclusion is that there are no reactive amino groups on adjacent molecules to the receptor which approach the FcR at the necessary distance (11-12 Å). A second possibility is that the FcR may need to be activated by an IgG dimer before an association exists with surrounding membrane proteins. Oimeric IgG is being developed now, as well as trimers, tetramers and so on, so that such a study can be performed. Such a procedure would be technically difficult but would be the next possibility to investigate. There is also a third possibility that the FcR near neighbor is not susceptible to probes of the intact membrane and upon solubilization for cross-linking of the cell extract, the neighboring molecule may lose its association with the FcR.

As mentioned in the introduction it is possible that the FcR functions alone in the process of endocytosis. In order to substantiate the negative findings from the studies done, cross-linking of Fc fragments of IgG-Woo and Fab fragments of goat anti-FcR to the FcR was attempted. By successfully cross-linking protein fragments of known molecular weight to the FcR and at the same time not being able to cross-link a neighboring molecule to the FcR, one could more appropriately conclude that the FcR is not associated with other molecules. Successful cross-linking of such fragments using DSS was not achieved however, but this may have been partially due to the incomplete lysis of the cells following cross-linking.
The 350,000 molecule isolated following cross-linking of intact cells and cell lysates with all of the cross-linking reagents, was conclusively identified as a dimer of the p170 molecule by this study. The p170 molecule is immunoprecipitated from U937 cells using goat anti-FcR\_II as a result of some nonspecificity of the antibody. The p350 molecule is thus not associated with the FcR.

Cross-linking reagents are more often than not difficult to work with but with continued perseverance and further experimentation these reagents may help to identify a subunit structure of the IgG FcR and thus aid in a more complete understanding of the endocytic process.
REFERENCES


APPENDIX

**Determination of ANB-NOS reactivity**

In order to determine whether the ANB-NOS synthesized by S. Isaacson in Dr. Anderson's laboratory in the summer of 1980 was still reactive, cross-linking was attempted with the ANB-NOS on a BSA solution of 1 mg/ml. The ANB-NOS was initially prepared at 400 mM in dioxane. Serial dilutions of 1:2 were then made for ANB-NOS solutions of 200 and 100 mM. 100 µl of each ANB-NOS solution was then added to 0.9 ml of the BSA to keep the dioxane at 10%. 100 µl of dioxane alone was added to one 0.9 ml BSA sample for a negative control. Final cross-linker concentrations were 10, 20 and 40 mM. Incubations and photolysis were as described in Methods. When the foil was removed from the sample tubes prior to photolysis, a yellow precipitate was observed in all tubes that contained ANB-NOS. This was probably due to excessive derivization of BSA by the NOS end of the reagent. This results in alteration of BSA tertiary structure with subsequent aggregation. The procedure was continued despite this observation and quenching of the NOS end was achieved with 0.5 M glycine/PBS. The samples were centrifuged and the supernatants prepared in LSB+ such that the SDS was at 2% and total sample volumes were 80 µl. Final protein concentrations of the samples loaded to a 5 to 10% gradient gel were 10, 31 and 41 µg. After staining and processing of the gel, double bands could be seen at a molecular weight of 68,000 (the molecular weight of BSA) in those samples that contained ANB-NOS. The sample without this reagent had a single band at 68,000. This may indicate that some intrachain cross-linking had occurred which altered the mobility of the protein slightly upon electrophoresis.

The results above did not provide convincing evidence that the ANB-NOS was still reactive. Thus another attempt was made to cross-link human IgG (HlgG) with the reagent. Cross-linking of IgG should result in more intrachain, then interchain
cross-linking and fewer problems with aggregation and precipitation should occur.

HlgG was prepared at 10, 50 and 100μg/ml and 3 1 ml aliquots of each were dispensed. One set containing each IgG concentration level was kept entirely in the dark. The other two sets were processed as above with one of the sets receiving dioxane alone. 110μl of ANB-NOS for a final concentration of 20 mM was used for cross-linking. When the foil was removed for photolysis of the appropriate samples, a yellow-white precipitate was again seen in tubes containing ANB-NOS. Less precipitate was seen with decreasing protein concentrations. The tubes were photolyzed, spun and the supernatants rephotolyzed. Both the precipitate and supernatants were analyzed. LSB+ was added to the precipitated material followed by boiling for two minutes. The material did not entirely dissolve but the supernatants were applied to the gel. TCA precipitations were performed on the original supernatants and LSB+ added to the TCA pellets. The TCA pellets did not completely dissolve either but were processed accordingly. This gel was not decipherable.

**Solubility testing of ANB-NOS in a suspension of U937 cells**

Since cellular material is not entirely protein it may be possible to cross-link U937 cells successfully with ANB-NOS without the solubility problems confronted with BSA and hlgG. Cell suspensions of U937 cells were prepared in PBS at 3.0 x 10⁶ cells/ml. ANB-NOS was prepared and added to 2.5 ml aliquots of these cell suspensions. Final ANB-NOS concentrations were 1, 3, 11, 33 and 100 mM. Precipitation was seen in the last two tubes while the remaining tubes appeared clear. These tubes were not examined microscopically however. Cross-linking of intact cells was then to be attempted using ANB-NOS concentrations of less than 33 mM.
Figure 8. General reaction mechanisms of a) imidates and b) N-hydroxysuccinimide esters with primary amino groups. c) Illustrates nitrene generation from aryl azides.

a) AMIDINE BOND FORMATION

\[
\begin{align*}
\text{NH}_2^+ \\
R - C - O - CH_3 + NH_2 - P & \to \ R - C - NH - P + CH_3OH
\end{align*}
\]

IMIDATE

b) AMIDE BOND FORMATION

\[
\begin{align*}
R-C-O-N & \xrightarrow{\text{N-HYDROXY-SUCCINIMIDE ESTER}} R-C-NH-P + HO - \\
\end{align*}
\]

NITRENE FORMATION

\[
\begin{align*}
\text{NITRENE} & \xrightarrow{\text{aryl azide}} \text{ARYL AZIDE}
\end{align*}
\]