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STUDIES ON ANTISERA TO RAT MAST CELLS AND RAT BASOPHILIC LEUKEMIA CELLS

BY

SUK HING YIU

A Thesis

Submitted to the Faculty of Graduate Studies in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy

Department of Immunology
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ABSTRACT

The present study is an investigation of the surface antigens of rat mast (RMC) and rat basophilic leukemia (RBL) cells. Anti-RMC and anti-RBL cell sera, three of each type, were induced in different rabbits by injecting them with the appropriate cells in Freund's complete adjuvant. All sera were decomplemented and were absorbed five times with rat liver cells. The anti-RMC sera were further absorbed with IgE-Sepharose. One antiserum of each group was selected for further studies based on its cytotoxicity to its target cells and its reactivity with the appropriate detergent-solubilized cell surface antigens. The two selected antisera, anti-RMC_{abs} and anti-RBL_{abs}, were not cytotoxic to rat lymph node cells but still killed RMC and RBL cells in the presence of complement. Binding between antibodies of the two sera and the surfaces of the two cell types was demonstrated by indirect immunofluorescence. Both antisera could induce skin reactions in normal rats and inhibit IgE binding to either RMC or RBL cells. In general, each antiserum was more reactive with its 'homologous' cells than with its 'heterologous' counterparts. Next, radioiodinated, detergent-solubilized surface antigens of either RMC or RBL cells were allowed to react with either antiserum. The immune complexes were insolubilized by Protein A-Sepharose and analyzed by SDS-polyacrylamide gel electrophoresis. The results showed that while anti-RMC_{abs} precipitated at least eight RMC surface antigens and mainly two RBL cell surface components, anti-RBL_{abs} reacted with at least

seven RBL cell surface antigens and about four RMC surface components. Furthermore, both antisera were shown to react with the 45,000 and 55,000 dalton receptors for IgE of RBL cells which had been adsorbed to and eluted from IgE-Sepharose. The above results indicated that both antisera contained antibodies to common surface antigens, including the receptors for IgE, shared by both RMC and RBL cells. Since both antisera were able to precipitate free receptors as well as IgE-receptor complexes, antibodies against more than one antigenic determinants of the receptor molecules may have been present. Additional studies, using both antisera which were absorbed with the cross-reacting cell type, revealed that both antisera contained antibodies to four other common antigens. Their molecular weights were in the range of 30,000 to 175,000 daltons. The above studies also revealed that anti-RMC_{abs} contained antibodies to one RMC-specific antigen (m.w. 85,000 daltons) whereas anti-RBL_{abs} had antibodies against one RBL cell-specific surface antigen (m.w. 78,000 daltons). The different amount of common surface antigens (as measured by cpm) bound by either antiserum indicated that not all antigens were equally immunogenic or equally expressed on the surfaces of RMC and RBL cells.

LIST OF ABBREVIATIONS

Anti-BSA	Serum, rabbit anti-bovine serum albumin
Anti-IgE	Serum, ε specific anti-rat IgE
Anti-RBL	Serum, rabbit anti-rat basophilic leukemia cells
$Anti\text{-}RBL_{abs}$	Serum, Anti-RBL absorbed with rat liver cells
$Anti\text{-}RBL_{mas}$	Serum, Anti-RBL absorbed with rat mast cells
Anti-RMC	Serum, rabbit anti-rat mast cells
$Anti\text{-}RMC_{abs}$	Anti-RMC absorbed with rat liver cells
$Anti\text{-}RMC_{bas}$	Anti-RMC absorbed with rat basophilic leukemia cells
Anti-S	Serum, rabbit anti-rat sarcoma cells
$Anti\text{-}S_{abs}$	Anti-S absorbed with rat liver cells
CFA	Complete Freund's adjuvant
cpm	Counts per minute
DNP	dinitrophenol
DNP-ONa	2,4-dinitrophenolate in 0.1 M phosphate buffer containing 0.1% NP-40
FCS	Fetal calf serum
F-IgE	Fluoresceinated immunoglobulin E
FITC-GAR	Fluoresceinated goat anti-rabbit immunoglobulin serum
HEPES	N-2-hydroxy-piperazine-N'-2-ethanesulfonic acid

IgE	Immunoglobulin E
IgG	Immunoglobulin G
L.N. cells	Rat lymph node cells
MES	2-(N-morpholino)-ethanesulfonic acid
m.w.	Molecular weight
NP-40	Nonidet P-40
NRS	Normal rabbit serum
NRS_{abs}	NRS absorbed with rat erythrocytes
PBS	Phosphate buffered saline (0.14 M NaCl, 0.01 M PO_4, pH 7.4)
PBS/BSA	PBS containing 0.05% bovine serum albumin
PBS/NP-40	PBS containing 0.1% or 0.5% NP-40
RBL	Rat basophilic leukemia cells
RBL(+)	A subline of RBL cells
R-IgE	Rhodaminized IgE
RMC	Rat mast cells
SDS	Sodium dodecyl sulfate
SDS-PAGE	Polyacrylamide gel electrophoresis in SDS
TEMED	N,N,N',N'-tetramethylethylenediamine
THM	Tyrode's buffer with 0.005 M HEPES and MES
THM/BSA	THM containing 0.05% bovine serum albumin
T lymphocytes	Thymus-derived lymphocytes

CHAPTER I

RAT MAST CELLS AND RAT BASOPHILIC LEUKEMIA CELLS

I. INTRODUCTION

Anaphylactic shock is a well established phenomenon in animals receiving an intravenous injection of a foreign protein. Histamine is one of the main chemical mediators for the reaction. Riley and West (1952) had first reported a consistent correlation between histamine content and mast cell number in a variety of tissues. Furthermore, the observation of mast cell degranulation followed by histamine release is well documented.

Asthma, allergic rhinitis (hay fever), eczema and urticaria are clinical examples of one type of immediate hypersensitivity. These allergies are quite common, affecting about 10% of the population. Immunologically, these atopic diseases are analogous to experimental animal anaphylaxis. Incubation of antigens with the blood or basophils of allergic individuals has been shown to result in liberation of histamine (Lichtenstein and Osler, 1964). The antibodies involved in these reactions are classically termed reagins or reaginic antibodies. Studies in recent years have identified these antibodies as a unique class of immunoglobulins designated as IgE (Ishizaka et al., 1966). The discoveries of human and rat IgE myeloma proteins have contributed greatly to the understanding of the structure and function of IgE immunoglobulin (Johannson and Bennich, 1967; Bazin et al., 1974, respectively). In addition, they provide opportunities for carrying out in vitro studies

on mechanisms of histamine release from mast cells or basophils. Ishizaka et al. (1970a) first showed the binding of radioactively labelled IgE myeloma proteins to the human basophil surface via the Fc portion of the molecule. Bach and Brashler (1973b) showed the specific binding of rat IgE to a cell free particulate preparation from rat peritoneal mast cells. Hence, the existence of a membrane-bound receptor for the Fc portion of IgE immunoglobulin in the target cells has been suggested. The demonstrations of histamine release from IgE-sensitized target cells in the presence of antigens (Lichtenstein and Osler, 1964) and anti-IgE antibodies (Ishizaka et al., 1969) have led to a postulation for the mechanism that triggers mediator release. It is believed that cross-linking between two cell-bound IgE molecules is essential for starting the secretion process. The membrane-bound receptor serves as an important intermediary for conveying signals between the exterior and the interior of the cell. Thus, membranes of mast cells and basophils and their receptors for IgE have become the subject of many studies not only because of their significance in allergy research but also as models for ligand-receptor interactions in secretory cells.

Since mast cells are readily obtained and can be enriched in populations from the peritoneal cavity of the rat, they are used for most of the studies on the mechanisms of histamine release. On the other hand, investigation of the secretion mechanism in basophils has suffered from one critical drawback. It has been technically difficult to isolate a pure preparation of normal blood basophils in relatively high concentrations. Studies of IgE binding to basophils have received a recent uplift as a consequence of the detection of IgE-binding receptors on a rat basophilic leukemia (RBL) cell line (Kulczycki et al., 1974). This

neoplastic basophil resembles the normal basophil in many ways (Chapter I, Section III:B). The most important advantage of this cell line is that receptors for IgE can be isolated from its plasma membrane (Conrad and Froese, 1976). It is no wonder that RBL cells have promptly become the main source of receptors for IgE since this cell line can be propagated by tissue cultures (Kulczycki et al., 1974).

II. ROLES OF RAT MAST CELLS AND RAT BASOPHILIC LEUKEMIA CELLS IN ALLERGY RESEARCH

The roles of rat mast cells (RMC) and rat basophilic leukemia (RBL) cells in studying the mechanisms for mediator release in immediate hypersensitivity are unique. The abundance of the receptors for IgE on the RBL cell surface (Chapter I, Section IV:B) has indirectly encouraged the development of techniques for isolating the receptor molecules from the membrane matrix. Free receptors (Conrad and Froese, 1978a; Isersky et al., 1978) as well as receptor-IgE complexes (Conrad and Froese, 1978b; Kanellopoulos et al., 1979) can now be purified and isolated by various methods. The availability of the purified receptor preparation has provided a great support for studying the structure and activity of the receptor for IgE. Details of these studies will be reviewed later. Since RBL cells are neoplastic cells, they cannot represent the true biological state of IgE-binding secretory cells found in their native environment. Therefore, the rat mast cell has inevitably become the 'referee' for most of the receptor-studies on RBL cells. Results obtained from these studies have to be validated by showing that they are also applicable to the studies of RMC. Only by doing so can the actual structure of the receptor for IgE and its related reactions be clearly understood. One of the aims of the present study is to find out the degree of antigenic cross-

reactivity between RMC and RBL cells. Details of the studies will be described in the next chapters. Meanwhile, attention is drawn to the morphological aspects of these two cells. Recent research in this area will be reviewed in the following section.

III. COMPARATIVE MORPHOLOGY OF RMC AND RBL CELLS

Although both mast cells and basophils have the same affinity for IgE immunoglobulin and both secrete histamine upon appropriate challenges, they are morphologically distinct from each other. Basophils are derived from bone marrow while precursor mast cells may originate in the thymus (Ishizaka et al., 1976). Humans have both blood borne basophils and tissue mast cells. In the rat, peritoneal fluid is rich in RMC while the blood does not have many basophils under normal physiological condition (Dvorak, 1978). The morphological distinction holds true also between RMC and RBL cells. In order to present a clear picture of how these two cells look, their morphology will be described separately and then compared.

A. Morphology of RMC

Rat peritoneal mast cells are generally spherical and have a mean diameter of 12.6 microns. The nucleus which is either round or kidney-shaped has a double-layered membrane and its presence is usually obscured by the abundance of the secretory granules. Only a few mitochondria are observed indicating that the mast cell has a low metabolic turnover. The endoplasmic reticulum is poorly developed, pointing to a low protein synthesis. Dense granules are seen near or within the Golgi Apparatus. The membrane is thin and is characterized by the presence of small villous processes. It is generally agreed that mast cells are at the end of a differentiation process since mitosis is rarely observed in these cells.

The mast cell is characterized by the presence of tightly packed granules. Each cell, on the average, contains some 500 granules (Uvnäs, 1964). The number and size of granules varies among different species. They stain metachromatically when a basic dye like toluidine blue is used, indicating the presence of sulfate compounds among their contents. The granules are relatively coarse in rat and mouse mast cells. They are either oval, round or irregular in shape as seen under the electron microscope. The secretory granules of rat mast cells contain several chemical mediators including histamine, heparin and serotonin. In addition, the presence of several enzymes like α-chymotrypsin, N-acetyl-β-D-glucosaminidase, peptidase, glycosidase has been described (Padawer, 1978). Rat mast cell granules contain zinc (Angyal and Archer, 1968) and iron (Pihl and Gustafson, 1967).

It has been established recently that rat peritoneal mast cells have the ability to phagocytize (Padawer, 1971). They can take up a wide variety of particulate substances including colloidal gold (Padawer, 1968), viruses (Padawer, 1971) and zymosan particles (Padawer and Fruhman, 1968). The binding between RMC and IgE immunoglobulin has been well documented. It is the events following the binding that generate much interest. Mast cells isolated from immunized rats will degranulate and release their chemical mediators when challenged with the specific antigen in vitro. It is also possible to sensitize rat mast cells from a normal animal by incubating the cells with reaginic antibodies or IgE myeloma protein. The sensitized cells can then be challenged with either the specific antigens or anti-IgE antibodies to release histamine. Uvnäs (1973) demonstrated that there was a correlation between the time that histamine is released and the time when rat mast cells degranulate. Studies with a low

molecular weight polymer, compound 48/80, showed that this compound triggers histamine release in rat mast cells. One of the first steps in rat mast cell degranulation induced by compound 48/80 involves fusion of the granular membranes with each other and with the surface membrane (Horsfield, 1965). There is indication that the fusion requires energy (Uvnäs, 1967). The fusion of membranes seems to lead to the formation of 'pores' within the cell membrane. As a result of a widening of these pores, the granules, now lacking their membranes, move freely to the exterior of the cell. Usually, a few seconds after the exposure of mast cells to the histamine liberator, granules are exteriorized by the cell. Most of the extruded granules adhere to their 'mother' cell thereby concealing the cell membrane. Uvnäs (1973) had tried to correlate morphological and biochemical events of antigen-induced histamine release from sensitized RMC. Mast cell granules consist mainly of a matrix of heparin-protein complex with the properties of a weak cation exchange resin, with COO^- groups as the cation binding sites. These amine-binding sites are exposed once the granules lose their perigranular membrane and come in contact with the extra-cellular fluid when the granules are extruded. Cation exchange takes place and consequently histamine and the other mediators are released in exchange for primarily sodium ions in the extra-cellular fluid. Histamine release from sensitized rat mast cells can be broadly divided into cytotoxic (immune complex-mediated) or non-cytotoxic (IgE-mediated) types. The IgE-mediated histamine release does not cause cell damage. It requires metabolic energy since metabolic inhibitors could block the release. Histamine release is also dependent on the present of Ca^{2+} and Mg^{2+} ions in the reacting system.

B. Morphology of RBL cells

Rat basophilic leukemia cells were first described by Eccleston et al. (1973). The initial tumors were developed in female Wistar rats which had been fed orally with β-chlorethylamine. Basophilic tumor cells were isolated from the tumors and were injected into new born Wistar rats for subsequent transplants (Kulczycki et al., 1974). These cells were later adapted to cell cultures (Kulczycki et al., 1974) and were shown to be morphologically similar to the tumor cells. The cultured cells vary in appearance from primitive cells resembling promyelocytes to well-differentiated basophils, depending on the growth conditions (Buell et al., 1976). Electron micrographs of RBL cells show that they have many of the features seen in normal human basophils, with no unusual features. Normal human basophils are generally spherical in shape with a diameter of 10 - 14 microns. They are the smallest of the granulocytes in blood. The nucleus of the human basophil is usually multilobed with a thin nuclear membrane. An electron microscopic view of a human basophil shows a small Golgi body with scattered cytoplasmic organelles like mitochondria, ribosomes and endoplasmic reticulum. The RBL cell surface consists of hundreds of villi as seen by the scanning electron microscope (Buell et al., 1976). The most striking appearance of a human basophil or a RBL cell is its cytoplasmic granules which can be stained metachromatically with basic dyes, e.g., toluidine blue and methylene blue. These granules are electron-dense, round, oval, and angular-shaped as well as being membrane-bound. Mature RBL cells contain secretory granules which can differ in sizes depending on the phase of growth of the cells in the culture. Buell et al. (1976) observed that during exponential growth of the cell, the granules were small. While the cells were at the stationary

phase of growth, basophilic granules increased in number and became more prominent. In the late stationary phase, these cells uniformly had the appearance of well-differentiated basophils with large granules. The granules generally decreased in size and number as the cells passed through successive divisions. Although granules of human basophils have been shown to contain chemical mediators, enzymes and metals similar to those present in mast cells (Padawer, 1978), no detailed studies have yet been carried out in RBL cell granules. Buell et al. (1976) reported that the histamine content per RBL cell remained rather constant despite rapid divisions that took place in the culture. It ranged between 0.6 - 0.9 $\mu g/10^6$ cells. The value is equivalent to that present in normal human basophils. The event of histamine release in basophils is also correlated to cellular degranulation. Dvorak et al. (1976) described the morphological changes of human leukemic basophils during degranulation. It began with fusion of granules, which was followed by fusion of their membranes with the plasma membrane, thereby creating open channels of communication between the granules and the extra-cellular space. This in turn permitted the sudden explosive release of granules. The sequential events described above closely resemble those associated with anaphylactic degranulation of rat mast cells. Exposure of the basophils of atopic patients to specific allergens in vivo or in vitro results in a prompt and explosive discharge of granules along with their contained mediators such as histamine and the entire event is generally completed within 5 - 15 minutes.

C. The Comparison

Morphological distinctions between mast cells and basophils have been described in full detail by Padawer (1978) and Dvorak (1978) separately. The difference mainly lies on the fact that the nucleus of the

basophil is segmented while that of the mast cell is not. Although granules of the basophil are metachromatic, their metachromasia is more labile than that of mast cells (Padawer, 1959). In humans, basophil granules are very water-soluble while mast cell granules are not. They also display different appearances, seen in the electron micrographs. The basophil granules are usually homogeneous or coarsely stippled in contrast to the complex and abundant whorls of the mast cell granules. Human mast cells are relatively long-lived, stretching from months to years, while human basophils have a life span of 8 - 12 days (Padawer, 1978). Rats and mice have very few basophils. Nevertheless, Dvorak (1978) claimed that rat basophils can be distinguished from tissue mast cells in terms of their nuclear and cytoplasmic structure. Rat basophils are smaller and rounder than mast cells. The cell surface of rat basophils has blunt cytoplasmic processes and short surface villi rather than the many, thin elongate villi typical of rat mast cells. In addition, the nucleus is generally bilobed or multilobed, in contrast to the mononuclear mast cells. The cytoplasmic granules are larger and less numerous than those of mast cells. Rat basophils, like human basophils, contain cytoplasmic glycogen, whereas rat mast cells have very little.

A detailed comparison between the morphology of RMC and that of RBL cells has not been reported. This could be due to the fact that the latter resemble normal basophils morphologically as well as cytochemically and they have been well compared with RMC in many ways. Furthermore, studies on RBL cells have been focussed mainly on investigating the nature of their receptors for IgE. Morphological and functional distinctions do exist between RMC and RBL cells despite their many similarities. Eccleston et al. (1973) found that RBL cells could ingest red cells in-

dicating a phagocytic function similar to that of RMC. They reported that neither alkaline phosphatase nor peroxidase could be found in either of these cells. Hence, the distinction between them lies on the degree of their metachromasia. According to their findings, although both types of cells stained metachromatically with toluidine blue, RMC stained more strongly. They claimed that the only staining method that could distinguish the two was Harada's method with chrysoidin. The mast cell granules consistently stained a bright yellow color while the basophil granules were negative. As far as the cytochemistry of the RBL cell granules is concerned, they have been shown to contain heparin, histamine and 5-hydroxytryptamine (Eccleston et al., 1973) similar to the mediators found in RMC. Recently, Jakschik et al. (1977) had described the release of a slow reacting substance from RBL cells. It could produce a prolonged contraction of guinea pig ileum and it was pharmacologically and chromatographically similar to that found in RMC. Despite all the similarities in their cytochemical contents, RBL cells and RMC have been shown to contain different secretion mechanisms. The first evidence came from Kulczycki's report (1974) that histamine release could not be induced in RBL cells with compound 48/80 or with antigen or anti-light chain antibodies using cells that had been pre-incubated with IgE. Rat mast cells can be induced to release histamine if treated the same way. Siraganian et al. (1975) found that anti-IgE antibodies failed to induce the release from IgE-bound RBL cells. While ionophore A-23187 could induce histamine release from RMC, a relatively higher but cytotoxic concentration was required in order to be effective for the induction in RBL cells. Although the induction was Ca^{++} dependent in both cell types, it could not be blocked by metabolic inhibitors when

RBL cells were used. On the contrary, histamine release from RMC in the presence of A-23187 is an energy dependent process. The accumulated findings seem to suggest that the RBL cells' unresponsiveness to IgE-mediated and histamine-liberator-mediated stimulation could be caused by a defect in one of the steps along the biochemical pathway leading to histamine release. More recently, it was discovered that a murine mastocytoma cell line was capable of releasing the mediators under non-cytotoxic conditions (Mendoza and Metzger, 1976a; Taurog et al., 1977). Originally this cell line was thought to be a mouse mastocytoma but it was later shown to be a subline of the RBL cells (Siraganian and Metzger, 1978). It will henceforth be designated as the RBL(+) cell.

IV. THE RECEPTOR FOR IgE

It was Ishizaka et al. (1970a) who first demonstrated the direct binding of IgE antibodies to the cell surface of human basophils using the technique of autoradiography. Later, they also showed the presence of IgE molecules on human skin mast cells (Ishizaka et al., 1971). Evidence that IgE binds to the membrane of the target cell was first presented by Bach and Brashler (1973b) who showed the specific binding between rat IgE and a cell-free particulate preparation from rat peritoneal mast cells. The membrane component that has an affinity for the IgE molecule has been referred to as the receptor for IgE. For the past five years, data obtained from the studies on this receptor have accumulated and they contribute greatly to the understanding of the structure and function of the receptor for IgE. Receptors can be visualized as the gateway for inducing the cellular secretion. The key role played by the receptor for IgE in immediate hypersensitivity is too significant to be neglected. The following is a summary of the findings.

A. Nature of the Receptor for IgE

Evidence that the receptor for IgE is membrane-bound came from the finding that its presence coincides with that of 5'-nucleotidase, a marker of the plasma membrane (König and Ishizaka, 1974). Since the receptor can be solubilized by non-ionic detergents (Conrad and Froese, 1976), it is reasonable to assume that this molecule has a hydrophobic region which is, most likely, buried deep into the membrane core. Furthermore, the presence of IgE on the surface of target cells can prevent the radioiodination of the receptor molecules (Conrad and Froese, 1976). This would indicate that only a small portion of the receptor molecule is exposed on the cell surface.

It is generally believed that IgE binds to the receptor via its Fc portion. The concept originated from indirect evidence given by Stanworth et al. (1968). Of all the papain and pepsin digested fragments of E myeloma protein which they tested, only the Fc fragment showed a significant blocking of the Prausnitz-Küstner reaction induced by human reaginic serum on human skin. Ishizaka et al. (1970b) provided the direct evidence by showing the binding of radiolabelled E myeloma protein to the human basophil surface via the Fc portion of the molecule. All the data indicate that the receptor has the affinity for the Fc fragment of IgE immunoglobulin.

The chemical nature of the receptor for IgE is still not clear. However, various studies indicate that the receptors are glycoproteins. Bach and Brashler (1973a) first showed that the binding between IgE and RMC was sensitive to the treatment of sialidase and phospholipase C. They suggested that sialic acid and β-linked galactose residues might play a role in the binding of IgE to RMC. The receptors were found to

be inactivated at temperatures above 37°C and at low (pH 2) or high pH (above pH 7) (Metzger et al., 1976). The receptor's sensitivity to temperature and pH changes indicates that part of its structural moiety is of protein in nature. The protein structure of the solubilized receptor is further suggested by the finding that it can be fragmented by trypsin (Conrad and Froese, 1976) and is sensitive to the presence of pronase and pepsin (Kulczycki et al., 1976). However, Metzger et al. (1976) reported that trypsin, chymotrypsin, pepsin and papain had no deleterious effect on the binding ability of their receptor when present in the membrane. They found that only phospholipase C and elastase could affect the binding activity. The facts that the receptors can be radioiodinated in the presence of lactoperoxidase (Conrad and Froese, 1976) and intrinsically labelled with ^{3}H-leucine (Kulczycki et al., 1976) provide additional evidence for the presence of tyrosine residues in the protein moiety of the molecules. Kulczycki et al. (1976) showed the biosynthetic incorporation of ^{14}C-glucosamine by RBL cells. Their result indicates that the receptors might contain sialic acid and glucosamines. In addition, the receptor was shown to bind to lentil-lectin-Sepharose and could be eluted from the affinity gel with α-methylmannoside (Helm et al., 1979). It was concluded that the receptor for IgE probably consisted of a carbohydrate moiety rich in mannose and/or N-acetylglucosamine, since the lectin is known to have specific affinity for these two sugars.

B. Specificity of the Receptor for IgE

It has been shown that radioiodinated monomeric IgG immunoglobulin does not bind to the surface of RMC (Ishizaka et al., 1975a) nor does it inhibit the binding between IgE and RBL cells (Kulczycki et al., 1974; Halper and Metzger, 1976). Although there is evidence that rat IgG_{2a}

can block the binding between IgE and RMC (Bach et al., 1971b), attempts to show direct binding between monomeric IgG_{2a} and RBL cells have not been successful. However, the binding of IgG_{2a} in the form of immune complexes to these cells has been demonstrated (Halper and Merzger, 1976). These results suggested that the affinity of IgG_{2a} for the receptor for IgE is not very high and that multipoint attachment is probably required for the binding to occur. Binding of monomeric IgE to other cell types has been reported (Gonzalez-Molina and spiegelberg, 1977; Meinke et al., 1978; Fritsche and Spiegelberg, 1978). However, the receptors on the surfaces of mast cells and basophils, including RBL cells, still remain the ones with high affinity for IgE.

The affinity constant of the binding between IgE and its receptor has been calculated. The equilibrium constant for the interaction between human basophils and IgE is at the order of $10^8 - 10^9\ M^{-1}$ and the process is reversible (Ishizaka et al., 1973). Kulczycki and Metzger (1974) demonstrated that the binding between IgE and its receptor on the surface of RBL cells is also reversible and can be described by the following equation:

$$\text{Receptor} + \text{IgE} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{IgE-receptor complex}$$

The association rate constant (k_1) between free IgE molecules and the receptors of RBL cells was $1.3 \times 10^5\ M^{-1}\ sec^{-1}$. The initial binding rate was dependent on the IgE concentration. The dissociation rate constant (k_{-1}) of the IgE-receptor complex was extremely low (1×10^{-5} - $1.35 \times 10^{-5}\ sec^{-1}$). The affinity constant for the binding was thus estimated to be around $10^{10}\ M^{-1}$. More recent findings showed that the cell-bound IgE dissociated from RBL cells with two different rate constants and that both the receptor and the IgE maintained their binding activity

after dissociation (Isersky et al., 1979). These results suggested that there may be two different receptors for IgE on RBL cells. The equilibrium constant for the binding between IgE and RMC was estimated to be $1.5 \times 10^{9}\ M^{-1}$ and the dissociation rate constant was believed to be low since no dissociation was observed over the first two hours (Conrad et al., 1975).

The very high affinity between IgE and mast cells/basophils confirms the specificity of the receptor. Furthermore, it has been estimated that both cell types contain a very high number of these specific receptors on their surface. There are approximately 40,000 to 600,000 receptors present on the surface of each human basophil (Ishizaka et al., 1973; Malveaux et al., 1978). The number of receptors for IgE on RBL cells was calculated to be around 600,000 to 1,000,000 per cell and the value is believed to be slightly lower in RMC, at 300,000 to 800,000 receptors per cell (Isersky et al., 1975; Conrad et al., 1975). In an extensive study on the changes in the number of receptors for IgE during growth and differentiation of RBL cells, Isersky et al. (1975) found that there was an inverse relationship between the growth rate and the expression of receptor activity for IgE. There were 400,000 to 600,000 receptors per RBL cell during rapid growth and the number increased to 900,000 to 1,000,000 when the cell stopped growth. Cell division resulted in a drop in the number of receptors per cell but the number of cell-bound receptors in the culture remained unchanged. This indicates that the receptors were simply divided up into daughter cells during mitosis. Furthermore, the continuous presence of IgE (3 μg/ml) in culture medium has been shown to have no effect on the expression of receptors by RBL-1 cells (Isersky et al., 1979). The relationship between expression of

the surface receptors and differentiation of the rat mast cell is not clear, although it was reported that the number of receptors for IgE does increase with mast cell maturation (Ishizaka et al., 1977a).

C. Isolation of the Receptor for IgE

Isolation and characterization of the receptors became possible when it was established that they could be solubilized by non-ionic detergents such as Nonidet P-40 without loss of their IgE-binding capacity (Conrad et al., 1976). The receptors for IgE were characterized by different laboratories using almost similar methods of isolation. The IgE-receptor complexes were initially isolated from the bulk of the membrane extract by simple immuno-precipitation using specific anti-IgE sera (Conrad and Froese, 1976; Kulczycki et al., 1976; Ishizaka et al., 1977b; Isersky et al., 1978). Radioactive isotopes like ^{125}I and ^{131}I were used invariably to label either the IgE or the membranes of the target cells. In one case, ^{14}C-glucosamine as well as ^{3}H-amino acids were also used for the study (Kulczycki et al., 1976). The possibility of obtaining the receptors in a relatively large quantity was first reported by Conrad and Froese (1978a) who described the isolation using IgE-affinity columns and KSCN as the eluting agent. Using repetitive affinity chromatography, Kulczycki and Parker (1979) were able to obtain relatively pure preparations of the receptors from RBL cell extracts. Hempstead et al. (1979) reported the isolation and characterization of the receptor for IgE from the surface of human basophils. Again, IgE-coupled Sepharose was used for the study.

Isolation of the free receptors by means of IgE-Sepharose columns has suffered from one critical drawback. Conrad and Froese (1978a) reported that only 19% of the eluted surface molecules had retained the

capacity to bind IgE. Rossi et al.(1977) could only recover 18% of the initial binding activity of their acid-eluted surface material. However, Kulczycki et al. (1979) used 0.5 N acetic acid to elute their receptors which still retained 50 - 60% of their original specificity. A third method with a potential for obtaining relatively pure receptors in the form of IgE-receptor complexes, using mild elution procedures, was developed by Conrad and Froese (1978b). These authors saturated RBL cells with DNP-IgE conjugates. The DNP-IgE-receptor complexes were then extracted with detergents and adsorbed onto an anti-DNP-affinity column and the IgE-receptor complexes were eluted with a buffer containing dinitrophenolate. The IgE-binding activity of this preparation had retained 50 - 60% of its original capacity. Based on the same principle, Kanellopoulos et al. (1979) used phenylarsonate-coupled IgE to saturate the receptors on the surface of RBL cells and subsequently characterize the isolated receptors.

D. Molecular Weight of the Receptor for IgE

The receptors for IgE have been isolated from different target cells and their molecular weights (m.w.) were subsequently determined by various methods. Initially, cell-free particulates containing IgE-binding activity were obtained from sonicated (at 20,000 x g) rat peritoneal mast cells (Konig and Ishizaka, 1974). Chromatographic analysis revealed that these fragments had an apparent m.w. of $> 2 \times 10^6$ daltons. In a separate study, cell-free particles containing the receptors for IgE were found present in the culture medium of RBL cells at $4^{\circ}C$ and their m.w. were reported to be at the same order of magnitude as the one above (Carson et al., 1975). The molecular weight of these particles suggested that they did not represent individual receptor molecules but were most likely

entire membrane fragments. Conrad et al., (1976) were the first to estimate the m.w. of Nonidet P-40 (NP-40) solubilized IgE-receptor complexes of RMC and RBL cells. When subjected to gel filtration on Bio-Gel A1.5m, the IgE-receptor complex was estimated to have an apparent m.w. of 350,000 to 550,000 daltons. After subtracting the m.w. of IgE (200,000 daltons), the m.w. of the receptor was in the range of 150,000 - 350,000 daltons. Using the same detergent but a different gel, Sepharose 6B, Rossi et al. (1977) determined the m.w. of the receptor and the IgE-receptor complex to be 250,000 and 410,000, respectively. The physical properties of the free receptor, the IgE-receptor complex and free IgE were determined and compared. Based on the values of their diffusion coefficients ($D_{20,\omega}$), partial specific volumes ($\bar{\upsilon}$) and sedimentation constants ($S_{20,\omega}$) in sucrose density gradient, the m.w. of the receptor, IgE-receptor complex and free IgE in NP-40 were calculated to be 130,000 310,000 and 200,000 daltons, respectively (Newman et al., 1977). Conrad and Froese (1978a) reported a m.w. of 200,000 daltons, or slightly less, for the receptor in NP-40 isolated by the IgE-affinity column and determined by gel filtration. Assuming that the receptor has a $\bar{\upsilon}$ of an average glycoprotein (0.72), Newman et al. (1977) calculated the m.w. of a detergent-free receptor to be 77,000 daltons. Also, based on the data that they obtained from this study, they postulated that the receptor molecule could be composed of two subunits of identical molecular weight. In addition, they concluded that the receptor is monovalent with respect to IgE binding.

When sodium dodecyl sulfate (SDS) was used to dissolve the receptor-IgE complex, subsequent determinations of the m.w. by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gave relatively lower values for

the receptors. The molecular weight of the free receptor was 62,000 daltons as analyzed by 5% SDS-PAGE (Conrad and Froese, 1976) and 70,000 daltons by 5.9% SDS-PAGE (Kulczycki et al., 1976). On 10 - 12% gels, the receptor molecule showed a faster mobility, revealing an electrophoretic behavior often associated with glycoproteins, and its molecular weight was estimated to be 50,000 daltons by Kulczycki et al. (1976) or 45,000 daltons by Conrad and Froese (1978a). This 45,000 dalton molecule was isolated by means of IgE, anti-IgE and Protein A-Sepharose. When IgE-Sepharose was used for the isolation of receptors from RBL cells, two IgE-specific surface molecules having molecular weights of 45,000 and 55,000 daltons were observed by SDS-PAGE on 10% gels (Conrad and Froese, 1978b). The 45,000 amd 55,000 dalton surface components (receptors) were later designated as R and H, respectively by Helm et al. (1979). On the other hand, although having both components expressed on its membrane surface, RMC seemed to be associated predominantly with the R component (Froese, 1979). Using also RBL cells, Isersky et al. (1978) estimated that the receptor for IgE had a m.w. of 58,000 daltons as analysed by 10% SDS-PAGE. On 12.5% gels, the same receptor was reported to have a m.w. of 53,000 daltons (Kanellopoulos et al., 1979). Even when IgE-Sepharose was used, only a single receptor of about 45,000 to 55,000 daltons was isolated (Kulczycki and Parker, 1979). In general, these results suggest that the RBL cell lines, maintained in the various laboratories, differ in spite of the fact that they all came from the original RBL cells induced by Eccleston et al. (1973). Recently, Hempstead et al. (1979) estimated that the m.w. of the receptor for IgE on the surface of human basophils was 58,000 - 68,000 daltons (10% SDS-PAGE).

On the whole, there seems to be a definite difference between the molecular weight of the receptor in the presence of non-ionic detergents and that in SDS as determined by SDS-PAGE. In fact, it has been reported that the physical properties of the receptors differed significantly between the presence of either one of the above (Conrad and Froese, 1978a). For instance, the receptor was shown to have a density of 1.24 g/ml in the presence of 0.1% NP-40 while the value increased to 1.28 g/ml in the presence of SDS. On the whole, the molecular weight of the receptor appears to be higher in the presence of NP-40. It has been postulated that this may be due to the binding of large amounts of NP-40 in the form of micelles, since the detergent concentrations are usually above the critical micelle concentration (0.29 mM) (Conrad and Froese, 1978a). These authors suggested limited aggregation of the receptor in NP-40 as an alternate explanation. Newman et al. (1977) postulated that the receptor probably consisted of two subunits while in NP-40. However, it should be pointed out that the capacity of the receptor to bind IgE is not impaired by the presence of NP-40. In fact, Rossi et al. (1977) reported that the binding constant for the receptor-IgE interaction was higher for the soluble receptor than for the membrane-bound one.

E. Function of the Receptor for IgE

Ishizaka and Ishizaka (1969) were the first to show that anti-IgE antibodies and their $F(ab')_2$ fragments can induce histamine release in human basophils of atopic individuals whereas their monovalent (Fab') fragments fail to do so. The results suggested that crosslinking of surface IgE molecules is essential for starting the triggering signal. Although the exact mechanism of triggering is not yet fully understood, it is likely that the receptors for IgE play an important role in the

event. Their possible function is indicated by the findings that they are fully mobile in the plane of the cell membrane. Using the technique of fluorescence photo-bleaching recovery, the diffusion coefficient of the receptors in the membrane was found to be $(2.1 \pm 0.5) \times 10^{-10}$ cm^{-2} S^{-1} (Schlessinger et al., 1976).

Surface IgE was seen diffusely distributed in the membrane of human basophils and redistribution of these IgE molecules could be induced when the cells were incubated with fluoresceinated anti-IgE antibodies (Becker et al., 1973). Patching of surface IgE could be observed when IgE-saturated RMC were incubated with fluoresceinated anti-IgE (Lawson et al., 1975). However, no capping was ever seen. Redistribution of the surface molecules has been found to be dose, time and temperature dependent. Capping occurred when human basophils or RBL cells were incubated with high doses of anti-IgE (10 - 20 μg/ml) at 37°C for 30 minutes (Carson and Metzger, 1974). It has been reported that gross aggregation of the surface IgE, i.e., capping, is not a pre-requisite for subsequent histamine release (Becker et al., 1973) whereas patching or clustering of the surface molecules has been seen just before degranulation in RMC (Lawson et al., 1975). In another study, dimerized rat IgE could induce anaphylactic skin reaction in normal rats (Segal et al., 1977). By the same token, anti-receptor antibodies could induce in vitro, non-cytotoxic mediator release from RBL(+) cells (Isersky et al., 1978) as well as RMC (Ishizaka et al., 1977b & 1978b). All the above data suggest that it is the bridging of the surface receptors for IgE that starts the triggering events. The exact number of receptor molecules involved is not yet known, although the data of Segal et al. (1977) suggested that two might be enough.

It is currently believed by some investigators that the receptor for IgE may consist of two identical subunits (Newman et al., 1977) or that it may exist in a multimeric form (Kulczycki et al., 1979). Using the technique of autoradiography, Ishizaka et al. (1975b) showed that occupied and unoccupied receptors for IgE could co-migrate in the membrane of human basophils. They suggested that the receptors were possibly linked together to form one functional group or that they were multivalent with regards to IgE binding. However, Mendoza and Metzger, (1976b) obtained a different result from their study. They saturated the target cells with both fluoresceinated IgE (F-IgE) and rhodaminized IgE (R-IgE) and allowed the cells to react with different antisera. When anti-IgE was used, comigration of F-IgE and R-IgE was observed. However, when the cells were treated with anti-fluorescein, they found that receptors binding R-IgE did not comigrate with those occupied by F-IgE. Hence, they concluded that individual receptors for IgE could function independently of the others and that each receptor was monovalent with respect to IgE binding.

It is still not clear exactly what kind of cellular changes might have taken place once when the receptors for IgE have been cross-linked. However, various models have been proposed for the initial events. One of these suggests that aggregation of the surface receptors can lead to a local conformational change of the membrane. This in turn could activate membrane-associated enzymes which would then turn on a chain of biochemical reactions leading to the ultimate release of histamine (Fewtrell and Gomperts, 1977). Another postulate is that aggregated receptors might themselves form ion channels allowing the transport of Ca^{++} across the membrane (Lawson et al., 1978). On

the other hand, aggregation may activate the receptor molecule which is believed to have an enzymic activity once activated (Fewtrell et al., 1979). It is obvious that there are many biochemical steps between the aggregation of the receptors and the release of mediators. Identification of the step immediately following the cross-linking of the receptors is particularly important since it will provide information to reveal the exact function of these molecules.

V. CELL SURFACE ANTIGENS

Individual cells can be distinguished on the basis of their surface morphology. Vital functions like growth and expansion, recognition of 'self' and 'non-self', cell movement, cell adherence, as well as uptake of nutrients are all manifested on the cell surface. Moreover, the functional diversity of different cell types is usually reflected by the distribution of different molecules present in and on the cell membrane. For instance, thymus-derived lymphocytes (T lymphocytes) have a distinct membrane antigenicity of their own as compared to bone marrow derived lymphocytes (B lymphocytes). Specific antisera, e.g., anti-thymocyte sera can lyse intact target cells in the presence of complement and are at the same time non-cytotoxic to unrelated cells (Stobo et al., 1973). This clearly indicates that different surface antigens are present in individual cell types. The immunological difference between normal and neoplastic cells generally lies in the presence of tumor-specific antigens on the surface of the latter.

The most important structure at the cell surface is the plasma membrane which is composed of amphipathic lipids and protein molecules (Danielli, 1975). Singer and Nicholson (1972) defined two different types of membrane proteins. Integral proteins are found tightly asso-

ciated with lipid bilayer. They are usually insoluble in aqueous solution but are soluble in non-ionic detergents. Proteins that are bound to the bilayer through ionic bonds or through other membrane components that are integral proteins themselves are generally termed as peripheral proteins and they are water soluble. Acetylcholine receptors and Fc receptors for IgG immunoglobulins are examples of integral proteins. Peripheral membrane proteins include cytochrome C and certain tumor antigens like the water soluble membrane antigens of P815 murine mastocytoma cells (Clemetson et al., 1976).

Membrane proteins, either integral or peripheral, as well as membrane carbohydrates can be immunogenic and antigenic. It is possible to specifically induce antisera against certain surface antigens of any cell type. Most of these studies indicate that surface determinants of unrelated cells from the same or related species exhibit cross-reactivities. However, tissue, organ or cell specific surface antigens do exist among the common antigenic determinants (Goldschneider and Moscona, 1972; Sell et al., 1969; Yiu and Froese, 1976; Behrens and Paronetto, 1978).

The surface antigens of murine lymphoid cells have been well studied and categorized. By comparison, rat cell membrane antigens are less well known. Nevertheless, scattered evidence has begun to accumulate. Besides, mice and rats are related species and their membrane antigenic determinants very often cross-react. For instance, rat and mouse liver-specific (Behrens and Paronetto, 1978) as well as T lymphocyte-specific (Ishii et al., 1976) cell surface antigens share some similar antigenic determinants. By combining some of the information obtained from murine cell studies with results from rat cell studies, one can obtain a better understanding of the antigens present on rat cell membranes.

A. Common Surface Antigens of Rat Lymphoid Cells

The most distinct specific antigens that are present on mammalian cell surfaces belong to the group generally referred to as histocompatibility antigens. The presence of these antigens on the cell surface usually determines the acceptance or rejection of tissue/organ transplants. For this reason they are also referred to as the transplantation antigens. They can be subdivided into three categories, the major histocompatibility, private and public, antigens and minor histocompatibility antigens. Their frequency in the population of a certain species determines whether the major histocompatibility antigens are private or public. These antigens are mainly responsible for tissue graft survival. Minor histocompatibility antigens are coded by genes outside the major histocompatibility complex (MHC) loci and are not as important in being responsible for the rejection of allografts. Antigens which are coded by the genes within the MHC have been extensively investigated. The H-2 complex of the mouse and the HL-A complex in man are well defined major histocompatibility antigens. By contrast, the rat histocompatibility system has only been studied and understood to a moderate extent. According to Palm et al. (1977), the MHC in rats consists of at least 20 histocompatibility loci that govern the expression of the transplantation antigens on the rat cell surface. The most common ones are the Ag-B or H-1 and Ag-C major histocompatibility complexes (Palm and Black, 1971). The H-1 locus can be further divided into two regions, H-1 A and H-1 B, as defined by possible recombinants (Butcher and Howard, 1977; Davies and Butcher, 1978). The H-1 A region has been shown to control lymphocyte and erythrocyte alloantigens, histocompatibility antigens and antigen targets for cytotoxic T lymphocytes. The H-1 B region

controls rat Ia antigens, histocompatibility antigens, antigens that stimulate strongly in the mixed lymphocyte reaction and genes governing the immune response to certain synthetic polypeptides (Cramer et al., 1974). One of the Ag-B antigens , Ag-B4, has been isolated from detergent-solubilized rat lymphoid cell membranes by affinity chromatography. The purified antigen was shown to have a molecular weight of 30,000 - 35,000 daltons (Callahan and DeWitt, 1975a). Amino acid composition of this antigen was found to be homologous to those of H-2 and HL-A antigens. Identification of several minor rat histocompatibility antigens has been reported. One of these, referred to as Ag-X, had a m.w. of 20,000 daltons (Callahan and DeWitt, 1975b). Williams and DeWitt (1976) identified another minor histocompatibility antigen present in rat lymph node cells and it was shown to be governed by the Ag-F locus situated outside the MHC loci. The AgF-1 antigen had a m.w. of 35,000 - 40,000 daltons. Its amino acid composition was roughly similar to those reported for the MHC antigens of the rat. The Ag-B4, Ag-X and AgF-1 antigens were shown to consist of a single chain structure since reduction and alkylation did not change their mobilities nor profiles on SDS-gels. By contrast, MHC antigens in mouse and man are believed to have a two-chain structure analogous to that found for immunoglobulins (Uhr et al., 1976; Strominger et al., 1974). The H-2 antigens of the mouse have a m.w. of 44,000 daltons and are usually found to be associated with β_2-microglobulins, m.w. of 12,000 daltons. While the structure of the MHC antigens in rats has yet to be defined, the presence of β_2-microglobulin on the surface of rat cells is established since anti-rat β_2-microglobulin serum can be induced successfully (Uhr et al., 1976). The I region within the H-2 complex of the mouse con-

trols Ia antigens which are found to be present in most lymphoid cells (Sachs and Dickler, 1975). The Ia antigen, m.w. of 30,000 daltons, has been isolated from the membrane of mouse lymphoid cells (Vitetta and Uhr, 1975), whereas rat Ia antigens have only been recently identified (McMaster and Williams, 1979). The rat Ia antigens were shown to be composed of two noncovalently linked polypeptide chains of apparent m.w. of 30,000 and 24,000 daltons. Apart from their own individually specific antigens, different cell types from the same animal or from the animals of the same strain/species would probably share some of the antigens mentioned above on their membrane surfaces.

In addition to the presence of the above antigens, rat lymphoid cells may also contain other common surface antigens. Most predominant of all is the group of leucocyte common antigens of m.w. 130,000 - 200,000 daltons (Standring et al., 1978). These differentiation antigens, of different m.w., were found to be present on the surfaces of rat thymocytes, periperal T and B lymphocytes as well as bone marrow cells, and they all reacted to the same anti-leucocyte common antigen serum. The presence of immunoglobulins on the surface of some rat lymphoid cells is well documented (Ishii et., 1974; Misra et al., 1976). It is likely that an antiserum directed against the surface antigens of a particular cell type might also contain anti-surface immunoglobulin antibodies. Recently, the occurrence of a surface antigen predominantly found in male rat cells has been reported. This male-specific antigen is referred to as the H-Y antigen which determines the fate of male skin isografts on female rats (Silvers et al., 1977). The carbohydrate structures on the cell surface are known to play a significant role in cellular interactions. The existence of 'natural' antibodies to membrane

carbohydrate components has been demonstrated in the classic studies of antibodies directed against blood group antigens. Specific anti-surface carbohydrate antibodies have been reported on separate occasions (Gerisch et al., 1974; Sela et al., 1975). These antibodies were shown to react predominantly with galactose, N - acetyl - D - glucosamine and N-acetyl-neuraminic acid, all of which were found to be present on the surface of cells of different tissues including the heart, kidney, thymus and spleen. Other undiscovered common antigens may be present on the rat lymphoid cell surface and are awaiting to be discovered.

B. Specific Surface Antigens of Rat Lymphoid Cells

This has not been an area widely studied. In fact, only a few specific antigens of rat lymphoid cells have been identified. Antigens of thymic specificity have been identified on the surfaces of thymocytes and peripheral T lymphocytes of the rat by different studies. Their m.w. was between 70,000 (Bustin et al., 1972; Misra et al., 1978) and 95,000 daltons (Standring et al., 1978). Another thymic antigen, Thy-1, has also been characterized. It is found mainly on the surfaces of thymocytes and brain cells of the rat and is believed to be composed of mainly protein and 32% carbohydrate (Williams and Standring, 1977). There are approximately 600,000 molecules of Thy-1 per rat thymocyte (Williams and Standring, 1977). The m.w. of Thy-1 was estimated to be 25,000 (Williams and Standring, 1977) to 27,000 daltons (Ladoulis et al., 1974; Misra et al., 1978). According to Williams and Standring (1977), there are at least three distinct antigenic determinants present in the Thy-1 molecule: (1) Thy-1.1 which cross-reacted with mouse anti-Thy-1.1 sera; (2) a rat-specific xenoantigenic determinant and (3) a rat-mouse cross-reacting xenoantigenic determinant. Antigens of other specificity have

not yet been fully characterized.

C. Specific Surface Antigens of Rat Mast Cells

Apart from common antigens like H-1 and non-H-1 antigens (Mossmann et al., 1976 and 1979), β_2-microglobulin (Isersky et al., 1977) as well as surface immunoglobulins (Austen and Humphrey, 1961), rat mast cells may also contain other specific surface antigens. Thus, the receptor for IgE may be a unique antigen of the rat mast cell surface (Yiu and Froese, 1976) as well as of the basophil surface. In addition, surface antigens, m.w. ranging between 20,000 to 200,000 daltons, have been shown to react with a specific anti-rat mast cell serum (Yiu and Froese, 1976). However, most of these antigens have not yet been fully identified.

D. Specific Surface Antigens of Rat Basophilic Leukemia Cells

Rat basophilic leukemia (RBL) cells were chemically induced (Eccleston et al., 1973) and may, therefore, contain individually specific tumor antigens. Malignant transformation is accompanied by alterations in the structure, topology and dynamics of plasma membrane glycoproteins (Nicholson, 1974). The effect of β-chlorethylamine on the membrane structure and antigenicity of RBL cells are still not clear. Although anti-RBL cell sera have been produced by different laboratories (Chapter I, Section VI:B), they were all used for the purpose of characterizing the receptor for IgE. As a result, tumor-specific antigens present on the RBL cell surface have not yet been identified. Whole membrane proteins have been extracted with NP-40 from radioiodinated RBL cells and were analyzed by 10% SDS-polyacrylamide gel electrophoresis (Helm et al., 1979). The RBL cell surface extract under reducing conditions was shown to contain at least ten membrane components with m.w. ranging from 17,000 to 200,000 daltons including components that were believed

to be reacting specifically with immunoglobulin E. The receptor for IgE is one of the antigens present on the RBL cell surface in relatively large numbers. Consequently, it is possible to induce specific anti-receptor antibodies (Ishizaka et al., 1977b; Isersky et al., 1978; Conrad et al., 1979). However, little is known about the nature of the other specific surface antigens of RBL cells.

VI. ANTISERA TO RMC AND RBL CELLS

There were three papers published before 1976 on antisera that were raised against rat mast cells. All three antisera were tested for their effects on the functions of mast cells, e.g., on inducing in vitro histamine release (Valentine et al., 1967; Hogarth-Smith and Bingley, 1971), on eliciting immediate skin reactions (Valentine et al., 1967) and on causing morphological changes (Smith and Lewis, 1961). Direct binding between the antibodies and RMC was demonstrated in only one case (Valentine et al., 1967) with an indirect immunofluorescent staining technique.

Another anti-rat mast cell serum was induced in 1976 with the purpose of studying the antigenicity of the receptor for IgE and mast cell surface antigens (Yiu and Froese, 1976). This antiserum was shown to be specifically cytotoxic to rat mast cells even after being absorbed five times with rat liver cells. It elicited an immediate skin reaction on rats when it was injected into skin sites. The absorbed anti-RMC serum strongly inhibited the binding between IgE and rat mast cells. Furthermore, the antiserum was shown to react with several membrane components of the same cell type with m.w. ranging from 20,000 to 200,000 daltons as revealed by 5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Using the same techniques, Conrad and Froese (1976) had previously de-

termined the m.w. of the receptor for IgE to be around 60,000 daltons. Among the mast cell membrane antigens precipitated by the anti-RMC serum and a goat anti-rabbit immunoglobulin serum, the receptor for IgE was likely to be present since one of the components had an apparent m.w. of 60,000 daltons. The results also indicated that antibodies against the receptor for IgE could possibly be induced.

During the course of the present study, techniques which have been developed over the past two years have made it possible to isolate free receptors and IgE-receptor complexes from the cell membrane preparation (Chapter I, Section VI:C). As a result, several anti-receptor sera were produced by various laboratories. The properties of these antisera will be described in the following text.

Isersky et al. (1977) first produced such an antiserum by injecting whole RBL cells emulsified with complete Freund's adjuvent into a rabbit. The whole antiserum, its IgG fraction and Fab fragments could inhibit the binding of IgE to intact target cells as well as detergent-solubilized membrane fragments of RBL and RBL(+) cells. Since the antiserum could precipitate free receptors as well as receptor-IgE complexes, it was suggested that the anti-RBL cell serum might contain antibodies to at least two discrete determinants in the receptor molecules. It was further postulated that one of the determinants was close to the combining site for IgE and the other was distal from the site. Soon after, the same group of authors reported the production of another anti-RBL cell serum by injecting a rabbit with partially purified receptor preparations (Isersky et al., 1978). This antiserum was found specific for the receptor for IgE but it also contained a fair amount of anti-IgE antibodies which had to be absorbed. The $F(ab')_2$ fragments were shown to

inhibit the binding between IgE and RBL cells. The antiserum could also induce the release of incorporated ^{3}H-serotonin from RBL(+) cells. In addition, it precipitated only one membrane component with an apparent m.w. of 58,000 daltons from the detergent extract of RBL cells. However, the same antiserum could precipitate at least two membrane components of the RBL(+) cell surface, with m.w. of 52,000 and 42,000 daltons, respectively. While anti-IgE precipitated only a 58,000 dalton component from the extract of IgE-saturated RBL cells, the identity of the smaller component was not clarified. In addition to the anti-receptor antibodies, the antiserum may have contained antibodies to other RBL cell surface antigens since IgE in excess had failed to inhibit the binding between the antiserum and the surface antigens completely. However, the nature of other RBL cell surface antigens was never studied in this work.

Ishizaka et al. (1977b) described the production of an antiserum by injecting a rabbit with preparations containing the immune complexes of anti-IgE and the receptor-IgE complexes. The antibodies were purified by adsorption onto $3 - 5 \times 10^{9}$ RBL cells and they were subsequently eluted from the cells with 0.1 M citric acid (pH 3) followed by neutralization of the antibodies. The purified antibody preparation could inhibit IgE binding to intact rat mast cells, RBL cells, as well as detergent-solubilized receptors. It could also induce immediate skin reactions in normal rats. The in vivo reaction was shown to be complement independent. The same antiserum could induce a non-cytotoxic histamine release only in RMC derived from inbred Hooded Lister rats. Immuno-fluorescent studies revealed that redistribution of surface antigens occurred when RBL cells were incubated with the antiserum and fluo-

resceinated anti-rabbit immunoglobulin antibodies at 37°C. If the cells were saturated with IgE before staining, the intensity of the staining would decrease. When the detergent-solubilized RBL or RMC cell surface extract was allowed to react with this antiserum and the immune complexes were subsequently analyzed with 10% SDS-PAGE, a major component with a m.w. of 45,000 daltons was revealed (Conrad et al., 1978). Conrad and Froese (1978a) had previously shown that the receptor isolated by IgE and anti-IgE has a m.w. of 45,000 daltons when analyzed by 10% SDS-PAGE. Since the antiserum prepared by Ishizaka et al. (1977b) reacted well with free receptors and only slightly with receptor-IgE complexes, it was concluded that the majority of its antibodies were mainly directed against determinants close to the IgE binding site (Conrad et al.,1978). The crude antiserum was shown to react with several other surface components of the RBL cell surface (Conrad et al., 1978) but the identity of the latter is not known.

More recently another anti-receptor serum was described by Conrad et al. (1979). The rabbit was injected with receptor preparations purified by means of IgE-affinity columns. The antiserum was shown to contain anti-receptor as well as anti-IgE activity and the latter was removed by absorption with IgE-coupled Sepharose. The specific antiserum could weakly inhibit IgE binding to RBL cells. Although being cytotoxic to these cells, it only faintly stained them by indirect immunofluorescence. However, the purified antiserum was shown to react with at least two RBL cell surface components of apparent m.w. of 55,000 and 45,000 daltons, respectively. These two components were demonstrated to be identical to the receptors isolated by means of IgE-Sepharose columns (Conrad and Froese, 1978a). The antiserum was shown to precipitate more of the

55,000 dalton component than the 45,000 dalton receptor. This finding indicates that the former surface molecule of RBL cells may be more immunogenic than the latter.

Studies on the properties of antisera to rat mast cells or rat basophilic leukemia cells have clearly demonstrated that these antisera serve as useful probes into the nature and properties of surface antigens like the receptor for IgE. In view of the fact that some of these did induce a non-cytotoxic mediator release from RBL(+) cells (Isersky et al., 1978) and rat mast cells (Ishizaka et al., 1977b), a new concept for the mechanism of target cell triggering has emerged. Bridging of two adjacent cell-bound IgE molecules on the membrane surface is no longer the only way to initiate the event of non-cytotoxic release of mediators. Cross-linking of two membrane receptors by anti-receptor antibodies can also trigger the release.

The anti-RMC or anti-RBL cell sera may play another role in understanding the nature of surface antigens of various target cells. Independent work by Mossmann et al. (1976) and Daëron and Voisin (1978) demonstrated that rat and mouse alloantibodies, respectively, could induce mediator release from rat and mouse mast cells, respectively. While antibodies against rat major histocompatibility antigens, H-1 antigens, could successfully inhibit the IgE-mediated release of histamine from rat mast cells, those directed against the non-H-1 antigens had failed to do so (Mossmann et al., 1979). Similarly, anti-rat β_2-microglobulin sera had inhibitory effects on the release (Isersky et al., 1977; Mossmann et al., 1979). The understanding of the relationship between H-1 antigens, β_2-microglobulin and the receptors for IgE still awaits further studies. In another report, cross-linking cell-bound IgE (anti-

DNP antibodies) with other cell surface components by DNP coupled to Fab' fragments of anti-mast cell antibodies also led to the subsequent release of histamine from rat mast cells (Mossmann et al., 1978). The nature of these mast cell surface components has not been revealed. On the whole, antisera directed against rat mast cells or rat basophilic leukemia cells may prove to be useful in certain studies. These studies may include the understanding of the structure, antigenicity and function of the surface components of these cells, especially, the receptor for IgE and their relationship with other membrane molecules.

CHAPTER II

CROSS-REACTIVITY BETWEEN ANTI-RAT MAST CELL AND ANTI-RAT BASOPHILIC LEUKEMIA CELL SERA

I. INTRODUCTION

The unique role of mast cells in immediate hypersensitivity has been discussed previously (Chapter I, Sections I, II & III). One of the most studied mast cell populations is the one present in the rat peritoneum. It owes its popularity to its availability in relatively large quantities. Although the involvement of rat mast cells (RMC) in allergy research has been well documented, there are still many questions left unanswered. For instance, the mechanism of triggering histamine release in RMC is not yet clearly understood. The emergence of rat basophilic leukemia (RBL) cells in allergy research has been timely. Although most RBL cell lines do not release histamine in a non-cytotoxic manner (Chapter I, Section III:C), the abundance of the receptors for IgE on their surface and the possibility to grow them in tissue culture helped significantly in the evaluation of the structure and function of the receptors for IgE. Thus, it has been shown that the receptors for IgE on RMC and RBL cells have very similar affinities for IgE (Conrad et al., 1975; Mendoza and Metzger, 1976a). The molecular weights of the receptors on both cell types are very similar (Conrad and Froese, 1976; Froese, 1979). Both cells have the 55,000 and 45,000 dalton components, even though less of the former appears to be present on RMC (Froese, 1979). However, in spite of the fact that antisera to RMC (Yiu and

Froese, 1976) and to RBL cells (Isersky et al., 1977) have been produced, very little is known about the antigens other than the receptor for IgE with which the antisera reacted. It is particularly not known to what extent plasma membrane antigens of the two cell types are similar to or different from one another. The availability of such information may eventually help to elucidate differences in the mediator release mechanism of the two cell types and to gain a better understanding of the relationship between cell surface molecules and certain functions of these cells.

It has gradually become apparent how the surface components of both RMC and RBL cells react to different ligands like IgE molecules, lectins, calcium ionophores and especially specific antibodies. The use of specific antisera to probe the nature of various membrane molecules, particularly receptors to hormones and certain chemicals like phosphorylcholine (Strayer et al., 1975) is now quite common. The production of anti-RMC and anti-RBL cell sera has been reported by various laboratories (Chapter I, Section VI). Since each antiserum is unique in terms of its properties and specificity, only by comparing and taking into account the accumulated data obtained from different studies can the nature of the surface of RMC and RBL cells be clearly understood. Hence, one of the aims of the present study is to produce these specific antisera. The similarities between RBL cells and RMC suggest that many of their surface molecules may be identical and hence, their specific antisera might possibly cross-react with the cells. Part of the present study is to look for such possibilities.

II. MATERIALS

Animals:

Female Lewis-Wistar rats, (retired breeders, 250 - 400 g), were purchased from Canadian Breeding Laboratories (Ottawa, Ontario). ICI Wistar rats were originally obtained from Imperial Chemical Industries, Ltd., Macclesfield, England and were later bred in the central animal house of the Faculty of Medicine of this university. Female New Zealand albino rabbits (3 - 4 Kg) were obtained from Canadian Breeding Laboratories (Ottawa, Ontario).

Buffers:

Tyrode's buffer was prepared according to the formula of Kabat and Mayer (1961) and was modified as suggested by Bach et al. (1971a) by making it 0.005 M with respect to both HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, and MES, 2-(N-morpholino)-ethanesulfonic acid. HEPES and MES were purchased from Calbiochem, Los Angeles, Calif. This buffer is referred to as THM and was adjusted to pH 6.8 for use. Phosphate buffered saline (0.14 M NaCl, 0.01 M PO_4; pH 7.4) was also used when required. Bovine serum albumin purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, was added to either THM or phosphate buffered saline (PBS) to yield a concentration of 0.5 mg/ml. These buffers are referred to as THM/BSA and PBS/BSA, respectively. Nonidet P-40 was purchased from Particle Data Corporation, Elmhurst, Ill. and was added to PBS to form a buffer called PBS/NP-40.

Antisera:

A rabbit anti-rat sarcoma (anti-S) serum was induced in an animal immunized with sarcoma cells from Lewis rats and was kindly provided by Dr. J. Dalton, previously associated with this department. Rabbit or

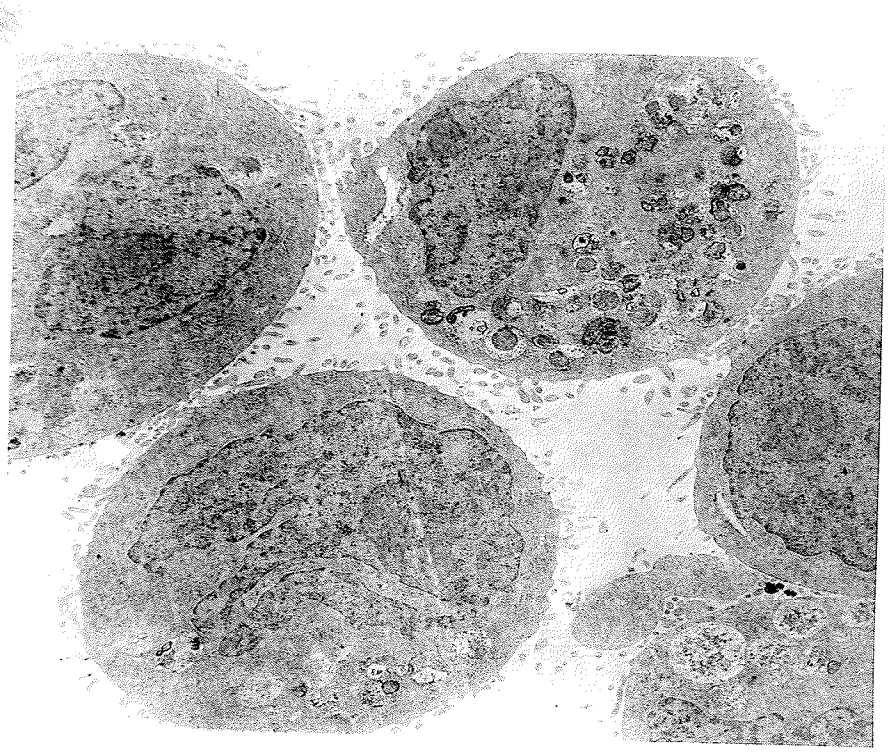
goat anti-rat IgE serum was prepared and described by Conrad and Froese (1978b) by immunizing the animals with purified IgE and absorbing the antiserum with normal rat serum coupled to Sepharose 4B. Goat anti-rabbit immunoglobulin serum (GAR) was purchased from North American Laboratory Supplies, Gunton, Manitoba.

IgE:

Rat monoclonal IgE was obtained from the ascitic fluid of Lou/M/Ws1 rats bearing the IgE-secreting IR-162 immunocytoma (Bazin et al., 1974). The tumor was maintained by Dr. B. G. Carter of this department. The IgE was routinely purified in Dr. A. Froese's laboratory by a combination of Bio-Gel P-300 chromatography and isoelectric focusing (Conrad et al., 1975).

Rat Basophilic Leukemia Cells:

The cells were routinely maintained by tissue culture in Dr. A. Froese's laboratory. The culture was initiated and maintained as described by Kulczycki et al. (1974) using Eagle's minimal essential medium, with Earle's base. The medium was supplemented with non-essential amino acids, 100 units/ml of penicillin, 100 µg/ml of streptomycin and 20% heat inactivated fetal calf serum (FCS). All ingredients of the culture medium were purchased from Grand Island Biological Co. (New York, N. Y.). All cultures were incubated at 37°C in a water saturated atmosphere containing 5% CO_2. An electron micrograph of these cells is presented in the following. This was prepared by the author as a study project in the course "Introduction to Electron Microscopy" given by the Department of Anatomy of this university. Since the present study does not involve the study of cell morphology, the picture is presented for the purpose of illustration only.



An Electron Micrograph of Rat Basophilic Leukemia Cells
(x 5,000)

III. METHODS

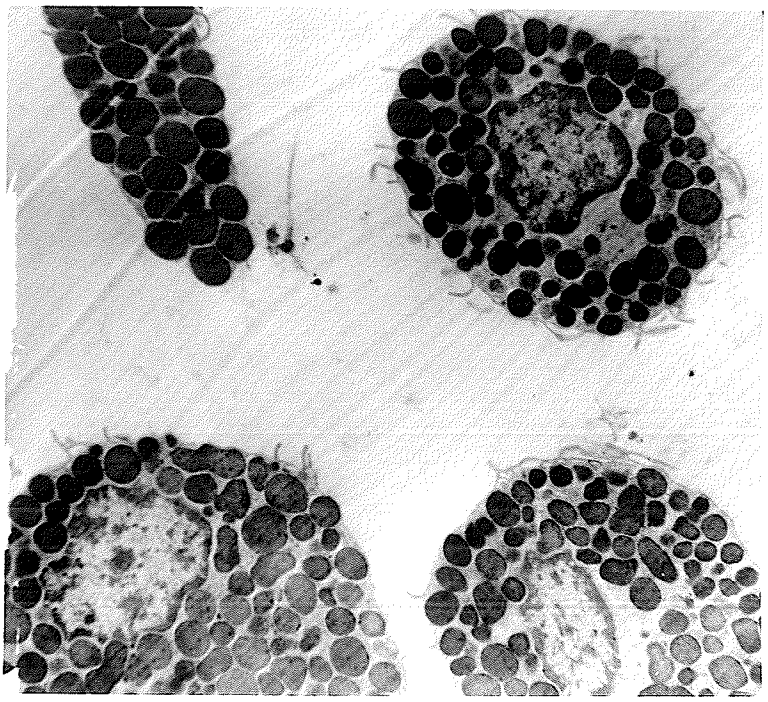
A. Purification of Rat Mast Cells:

The procedures are mainly based on the technique of Uvnäs and Thon (1959). The principle of the method involves the layering of the peritoneal cell suspension over a high density medium. Centrifugation is used to force the denser mast cells through the interface and these may be collected in the high density medium. Macrophages and erythrocytes are retained above or at the interface.

Rats were exsanguinated under ether anesthesia and the skin along the mid-ventral part of the body was removed. About 20 ml of THM/BSA containing 10 units/ml of heparin (ICN Pharmaceuticals, Inc., Cleveland,

Ohio) were injected into the peritoneal cavity of the the abdomen which was gently massaged for 1 - 2 minutes. A small incision was made just large enough to admit the passage of a plastic tube (12 x 75 mm, Falcon Plastics, Oxnard, California) the end of which had been perforated several times with a hot needle. The tube was inserted deep into the abdomen and the peritoneal fluid was collected with a siliconized Pasteur pipette and transferred into a polycarbonate centrifuge tube (2.7 x 10.5 cm). The cells were then centrifuged at 200 x g for 5 minutes. The pellet was resuspended in 4 ml of THM/BSA buffer and cell counts were performed with a hemocytometer (American Optical Corporation). Mast cells were identified by staining with neutral red which formed a dry film on the cover slip of the hemocytometer (0.2% neutral red in ethanol). The initial suspension contained 5 - 10% mast cells. The cell suspension was then layered on top of an equal volume of Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden), (35% Ficoll, w/v, in THM/BSA), in a polycarbonate centrifuge tube and was spun at 200 x g for 2 minutes at 4°C. At the end of this time, the upper layer and cells at the interface were removed. The tube wall around the area was wiped clean with a piece of lint-free tissue paper. About four times as much THM/BSA was added into the Ficoll medium which now contained mainly mast cells. The tube was centrifuged at 200 x g for 5 minutes and the cell pellet was resuspended with THM/BSA. Then, the cells were stained and counted. The count was expressed in % of purity (={number of mast cells/total number of cells} x 100) and % of yield (={final number of mast cells/initial number of mast cells} x 100). Approximately 60 - 75 percent of the mast cells were recovered in the final preparation and the purity was between 85 - 90%. An electron micrograph of rat peri-

toneal mast cells is presented in the following text. Like the one of RBL cells, this photograph was prepared for the purpose mentioned in the previous section.



An Electron Micrograph of Rat Mast Cells
(x 4,000)

B. Preparation of Antisera:

Two groups of rabbits, three animals per group, were injected with isolated rat mast cells (RMC) or with rat basophilic leukemia (RBL) cells. Mast cells (1×10^7) from five rats were washed extensively with THM after the Ficoll separation procedure and were suspended in 1 ml of THM. The mixture was then emulsified with an equal volume of complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, Michigan) and was injected subcutaneously onto the shaved lateral parts of

the rabbit. Three rabbits were injected with RMC this way at separate occasions while another three were immunized with RBL cells (3×10^7 per animal) instead. The latter had been injected with RBL cells that came from the same pool of culture and were washed thoroughly with PBS by centrifugation before being emulsified with CFA. About thirty days after the first injection, the rabbits were injected with a second dose. The number of cells used was 5×10^6 for RMC or 1×10^7 for RBL cells. The cell suspensions were emulsified with CFA and were then injected the same way as before. The third and fourth doses of the same strength as the second one were given about 14 and 28 days, respectively, after the second injection.

Blood was obtained from the marginal ear vein of the rabbit and the serum was separated out by allowing the blood to clot, followed by centrifugation at 1000 x g for 20 minutes at $4^{\circ}C$. All sera were heated at $56^{\circ}C$ for over thirty minutes and were filtered through Millipore membranes (0.22 μ) and stored in sterile vials at $4^{\circ}C$. The serum was withdrawn from the vial with a sterile needle and syringe when required. A crude immunoglobulin preparation was made as required by precipitation with 40% saturated ammonium sulfate. The precipitate was washed twice with PBS containing 40% saturated ammonium sulfate followed by centrifugation at 10,000 rpm for 20 minutes. It was then dissolved in a small volume of PBS and dialyzed against several changes of PBS at $4^{\circ}C$. After dialysis, the sample was reconstituted to its original volume. The protein concentration of the sample was determined by measuring its optical density and from the result calculated by using an extinction coefficient of 13.6 ($E^{1\%}_{280\ nm}$). The preparation was then stored at $-20^{\circ}C$ for further use.

C. Isolation of Cells:

1. Rat liver cells

Rat livers were removed from exsanguinated rats, rinsed with cold PBS, and were minced and disrupted with a loosely fitted glass homogenizer. The dispersed cells were suspended into cold PBS and filtered through Pyrex glass wool. The preparation was washed with cold PBS by centrifugation at 200 x g for 10 minutes at least three times.

2. Rat lymph node cells

Cervical lymph nodes were removed from exsanguinated rats, rinsed with cold PBS and were treated the same way as described above. The cells were then suspended in THM/BSA supplemented with 10% fetal calf serum for subsequent use.

3. Rat erythrocytes

Blood was collected with a heparinized syringe containing a small volume of Alsever's solution, prepared according to the formula of Campbell et al. (1970), from anaesthetized Lewis-Wistar rats via cardiac puncture. The blood was mixed gently and washed with an equal volume of Alsever's solution and centrifuged at 700 x g for 20 minutes. The washing procedure was repeated until the supernatant was clear.

4. Mast cell-depleted rat peritoneal cells

These cells were obtained by the method previously described by Bach et al. (1971a). Ten milliliters of distilled water were injected intraperitoneally into rats. Four days later, the peritoneal cells were harvested the same way as described in Section III:A of this chapter. The cell preparations were examined under the light microscope for the presence of mast cells. Usually only about 1% mast cells remained in the entire peritoneal cell population.

D. Preparation of Sepharose Conjugates:

This was a slightly modified method as described by Cuatrecasas and Anfinsen (1971). Ten ml of washed, packed Sepharose-CL-4B were activated with cyanogen bromide (150 mg/ml of packed gel) at pH 11. The activated gel was washed with a large volume of cold distilled water followed by cold PBS on a Buchner funnel. The gel was then transferred into a small beaker containing 10 ml of protein to be conjugated at a concentration of 14 - 15 mg/ml. The coupling reaction was carried out at 4°C for 24 hours. The immunosorbent was washed with PBS until the filtrate was free of protein and it was then transferred into a beaker. Any unreacted Sepharose-CL-4B was inactivated by adding 50 ml of 0.05 M ethanolamine at 4°C and the reaction was allowed to proceed for thirty minutes. The gel was then washed with PBS again, followed by 200 ml of 0.2 M glycine-HCl buffer (pH 2.2) and PBS until the filtrate remained at pH 7 and had no trace of protein as determined by its optical density at 280 nm.

E. Absorption Procedures:

1. Cell-absorption

About 0.1 ml of packed cells was used for each ml of diluted serum (1 : 10). The mixture was incubated at 4°C for one hour with constant rotation. At the end of each absorption, cells were removed by centrifugation at 200 x g for 10 minutes. The procedure was repeated several times, if required. The serum was then clarified by centrifugation at 50,000 x g at 4°C for one hour.

2. Immunosorbent-absorption

Sepharose conjugated with immunoglobulins was used as the immunosorbent in this study. It was packed into a small glass column (14 x

110 mm) with its outlet filled with glass beads (3 mm in diameter). The column was washed well with PBS. The globulin fraction of the serum to be absorbed was layered gently on top of the packed immunosorbent. The absorption was carried out at room temperature and the flow was adjusted to one drop per 30 seconds. After the sample had passed through the gel, the column was topped up with PBS. About 1 ml of the eluate was collected into each tube (9 x 75 mm). The absorbance of each fraction was measured at 280 nm. Fractions having an optical density higher than 1 were pooled together. The pooled eluate constituted the absorbed serum. The absorbed serum was then filtered and stored in sterile vials at $4^{o}C$.

F. Cytotoxicity Test:

The test was performed according to the method of Wigzell (1965) as modified by Fujimoto et al. (1973). Target cells (1×10^{7}) were suspended in THM to which had been added 10% heat inactivated fetal calf serum and were labelled with 0.1 mCi of $Na_2(^{51}Cr)O_4$ (Amersham-Searle Co., Arlington Heights, Illinois) at $37^{o}C$ for 30 minutes. After labelling, the cell suspension was gently layered on top of 2 ml of fetal calf serum followed by centrifugation at 1000 rpm for 1 minute. The supernatant was carefully aspirated and the cell pellet was resuspended with the same buffer. The cells were washed by centrifugation at 200 x g for 8 minutes. The cell pellet was resuspended with 10 ml of the medium to yield a concentration of 10^{6} cells per ml. Sera to be tested were diluted with the same buffer directly on the tissue culture plate (Microtest II, Falcon Plastics, Oxnard, Calif.). To 0.1 ml of serum in each well of the plate, 0.1 ml of the cell suspension was added, followed by 0.1 ml of complement (diluted 1 : 10). Guinea pig complement was used when lymph node cells and RBL cells

were the target cells. Fresh rabbit serum served as the source of complement for the test with mast cells. The plate was sealed with a piece of microtiter plate sealer (Cooke Engineering Co., Alexandria, Virginia) and was incubated at 37°C for 30 minutes. At the end of this time, the plate was centrifuged at 200 x g for 10 minutes. The radioactivity of 0.1 ml of supernatant from each well was measured in a Beckman gamma counter (Model 300). Controls were performed under the same conditions at the same time. The maximum release of ^{51}Cr was arbitrarily determined by freezing and thawing 10^5 target cells in distilled water three times and then counting the radioactivity of the supernatant. The presence of ^{51}Cr in supernatants from labelled cells in the presence of complement alone was taken as the value for the spontaneous release. Duplicate samples were performed for each test. The results were obtained in counts per minute (cpm) and were expressed as a percentage of specific ^{51}Cr-release which was calculated with the following equation:

Percentage of specific ^{51}Cr-release = { (experimental release - spontaneous release) / (maximum release - spontaneous release)} x 100

G. ^{125}I-labelling of IgE:

Rat monoclonal IgE purified as described by Conrad et al. (1975) was labelled according to McConahey and Dixon (1966) using chloramine T. Purified IgE (100 µg in 0.05 ml of PBS) was labelled with 1 mCi of carrier-free ^{125}I (Amersham-Searle, Arlington Heights, Illinois) in the presence of 0.45 ml of PBS and 0.25 ml of chloramine T (25 mg / 100 ml of PBS). The reaction was allowed to proceed at room temperature for 5 minutes with occasional mixing. An addition of 0.3 ml of sodium metabisulfite (25 mg / 100 ml of PBS) stopped the labelling reaction.

The labelled protein in solution was mixed with 250 mg of Dowex resin (AG1-X10, Bio. Rad., Richmond, California) washed with PBS. The mixture was allowed to stand at room temperature for one minute and then was centrifuged at 250 x g for 10 minutes. The supernatant was removed and dialyzed versus 2 litres of PBS overnight. The optical density of the labelled protein was measured and hence, its concentration determined by using an extinction coefficient of 13.6 ($E^{1\%}_{280\ nm}$) (Carson and Metzger, 1974). The ^{125}I-IgE solution was adjusted to a concentration of 1 µg per ml with PBS/BSA and was stored in aliquots at -70°C. The specific activity of the labelled IgE was in the range of 2 - 6 x 10^6 cpm per µg. The counting was done in a Beckman gamma counter (Model 300, Beckman Instrument Co., Fullerton, California) with a counting efficiency of 55% for ^{125}I.

H. Precipitation of ^{125}I-IgE by Antisera:

About 0.2 mg of rabbit immunoglobulins of the antisera were mixed with 0.05 µg of ^{125}I-IgE and the mixture was incubated at 37°C for 30 minutes. A predetermined optimal amount of goat anti-rabbit immunoglobulin was added and the reaction continued for another 30 minutes. The immunoprecipitate was allowed to form at 4°C over a period of three to four hours. The precipitates were transferred to clean tubes and were washed five times with PBS through centrifugation. The radioactivity of each precipitate was then determined. Normal rabbit immunoglobulins and rabbit anti-rat IgE antibodies were used in place of the antisera as controls. The results were recorded in cpm and were expressed as the concentration of IgE precipitated by the corresponding serum.

I. Inhibition of ^{125}I-IgE Binding to RBL Cells or RMC:

The method was adopted from the one described by Kulczycki et al. (1974) as modified by Conrad et al. (1975). Purified rat mast cells (RMC) or rat basophilic leukemia (RBL) cells, at a concentration of 1×10^6 in 1.0 ml of THM/BSA, were incubated with 400 µg of rabbit immunoglobulins from the antisera in silicone grease coated plastic tubes (12 x 75 mm, Falcon Plastics) at 37°C for 30 minutes. The coating prevented adherence of the cells to the plastic wall. At the end of the time, 0.1 µg of ^{125}I-IgE was added into each tube and the incubation was continued for another thirty minutes. The cell suspension was layered gently on top of 1.5 - 2 ml of FCS and was centrifuged at 250 x g for 1 minute. The cell pellet was recovered and washed once with THM/BSA by centrifuged at 200 x g for 8 minutes before the ^{125}I content of the pellet was determined. Cell pellets obtained from the cells incubated with ^{125}I-IgE and THM/BSA in the absence of any antiserum were used as controls for the maximum binding of IgE. The averaged value obtained from these controls became the arbitrary 100% of IgE binding . Each result was recorded in cpm and was expressed as a percentage of inhibition of IgE binding according to the calculation of the following formula:

$$\% \text{ of inhibition} = 100\% - \frac{\text{cpm of experimental result}}{\text{cpm of maximum IgE binding}} \times 100\%$$

J. Cell Surface-Labelling Technique:

Cells were radioiodinated according to the method described by Kennel et al. (1973) as modified by Conrad and Froese (1976). Cells (5×10^6 RMC or 2×10^7 RBL cells in 0.5 ml of PBS) were labelled with 0.5 - 0.7 mCi of carrier-free ^{125}I (or 1 mCi of carrier-free ^{131}I) in

the presence of 0.01 ml of lactoperoxidase (60 mg/ml). The reaction was started by adding 0.01 ml of 0.03% H_2O_2. The addition of H_2O_2 was repeated twice at one minute intervals. One minute after the third addition, the reaction was stopped by transferring the cell suspension into 10 ml of cold THM/BSA followed by centrifugation at 200 x g for 5 minutes. The cells were resuspended in 1 ml of THM/BSA and were then layered on top of 1.5 - 2 ml of FCS for the same washing procedure as mentioned in Section III:F of this chapter. The washed cells were solubilized with 1 ml of PBS/0.5% NP-40 at 4°C for 15 minutes. The cell mixture was centrifuged at 10,000 rpm for 15 minutes in a Sorvall RC2-B centrifuge (Ivan Sorvall Incorporated, Newtown, Connecticut). The supernatant was recovered and dialyzed versus PBS/0.5% NP-40 overnight.

K. Precipitation of Radiolabelled-Cell Surface Antigens:

The cell surface extract obtained by the above procedure was divided into two aliquots. When more cell extract was required, several batches of cells were labelled and their detergent-solubilized surface material was then pooled together and redivided as required. Each aliquot of the above was incubated with a known amount of rabbit immunoglobulins (0.4 mg per sample) from the antisera or from normal rabbit serum at 37°C for thirty minutes. When anti-IgE was used, 0.01 mg of IgE was first of all incubated with the cell extract at 37°C for 30 minutes, then the anti-IgE (0.4 mg) was added, after which an additional 30 minutes of incubation followed. A predetermined amount of goat anti-rabbit immunoglobulin serum (GAR) was added to the above sample and the incubation was carried out at 37°C for another 30 minutes. The immunoprecipitate was allowed to form as described in Section III:H of this chapter and was subsequently dissolved in 0.1 - 0.2 ml of Tris buffer

(1 M, containing 2% sodium dodecyl sulfate, 9 M urea, pH 8.5) at 37°C for three hours. The solubilized precipitate was then dialyzed against 0.01 M phosphate buffer containing 0.1% sodium dodecyl sulfate (SDS) and 0.5 M urea at pH 7.2 overnight. The sample was ready for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 5% gels. Alternatively, 0.1 ml of packed Protein A-Sepharose (Pharmacia, Uppsala, Sweden) was added into the sample instead of GAR and was mixed on a test tube shaker (Model G-33-A, New Brunswick Scientific, New Brunswick, N. Y.) at 250 rpm for 90 minutes at 4°C. The supernatant was removed by centrifugation and the Sepharose beads were washed with PBS/0.1% NP-40 four times, followed by washing with 0.0625 M Tris HCl, pH 6.8 once. The bound material was eluted from Protein A-Sepharose by dissolving in 0.2 ml of the above buffer in the presence of 2% SDS at 100°C for 90 seconds. The supernatant was removed carefully and was ready for analysis by SDS-PAGE on 10% gels.

L. SDS-PAGE Analysis:

1. On 5% gel

The samples were analyzed according to the method described by Shapiro et al. (1967). Glass tubes 10.5 cm long with an inner diameter of 6 mm were coated with a 1% Column Coat solution (Canalco, Rockville, Maryland). For a typical run of 4 gels, a mixture of 1 ml of 0.1 M phosphate buffer with 1% SDS and 5 M urea at pH 7.4 and 2.5 ml of a 20% acrylamide in 0.5% methylene-bisacrylamide solution was deaerated. This was followed by an addition of 0.5 ml of a freshly prepared 2% ammonium persulfate solution, 5 µl of N,N,N',N'-tetramethylethylenediamine (TEMED) and 7 ml of distilled water. Each tube was filled with 2.5 ml of the above mixture and gels (9 cm long) were left to hard-

den. A drop of tracking dye (0.05% Bromophenol blue in water) and a drop of glycerine were mixed with each of the dissolved immunoprecipitates. The mixture was then layered on top of the gel and the tube was filled with the electrode buffer. The latter was a 0.01 M phosphate buffer containing 0.1% SDS and 0.5 M urea at pH 7.2. The upper and the lower reservoirs of the electrophoresis apparatus were filled with the same buffer mentioned above. The gels were subjected to electrophoresis at 8 mA per gel. The process was discontinued when the tracking dye reached the bottom of the gel. The gels were then sliced on a Gilson Model B-200 gel fractionator (Gilson Medical Electronics, Inc., Middleton, Wisconsin) into 2 mm fractions and their radioactive contents were measured.

2. On 10% gel

This was performed with the Tris-buffered system as described by Laemmli (1970). Gels containing 10% acrylamide were prepared from a stock solution of 30% by weight of acrylamide and 0.8% by weight of N,N'-bis-methylene acrylamide (Bis). The final concentrations in the separation gel were as follows: 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS. The gels were polymerized chemically by the addition of 0.025% by volume of TEMED and ammonium persulfate. The separation gel had a length of 14 cm and the stacking gel (3% acrylamide) had a length of 1 cm. The stacking gel also contained 0.125 M Tris-HCl (pH 6.8) and 0.1% SDS and was polymerized the same way as mentioned above. Usually the 10% gel was poured and allowed to set before the 3% gel was layered on top. The sample to be analyzed, in the sample buffer, was then layered on top of the gel. Stacking of the sample was performed at 1.3 mA per tube while separation was carried out at 3.3 mA per tube. The elec-

trode buffer used for this system contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS at pH 8.3. When the tracking dye had reached the bottom of the gels the run was stopped and the gel was sliced as mentioned previously. The radioactive content of each fraction was measured in a Beckman gamma counter.

When both ^{125}I and ^{131}I were present in the sample, the ^{131}I window of the gamma counter was adjusted so as to eliminate most of the ^{125}I counts (less than 0.1%). The cpm in the ^{125}I channel were corrected for ^{131}I spillover as described by Gaze et al. (1973). All results were expressed graphically by plotting cpm versus fraction number starting from the top of the gel.

M. Indirect Immunofluorescence:

Antibodies against RMC and RBL cells were determined by indirect immunofluorescence. Unless specified, the treatment of the cells with antibodies was carried out at $4^{\circ}C$ in THM/BSA containing 10 mM sodium azide. Cells (1×10^5 in 0.1 ml of buffered medium) were incubated for 30 minutes with an equal volume of antiserum or normal rabbit serum diluted to a known concentration. Cells were washed three times and resuspended in 0.1 ml of the medium. An equal volume of an 1 : 10 dilution of fluoresceinated goat anti-rabbit immunoglobulin serum (FITC-GAR) (Miles laboratories, Kankakee, Illinois) was added to the cell suspension which was then incubated for another 30 minutes. Cells were washed three times and were resuspended in 0.1 ml of the medium containing 20% glycerol. Fluorescent staining was examined with a Leitz Ortholux microscope, equipped with an Osram HB 200 mercury lamp, BG 38 and KP 490 excitation filters, dark field condenser , and K510 plus K530 suppression filters. About 100 cells in each cell prepara-

tion were examined for surface staining. The results were recorded as positive or negative staining and the intensity of the staining was scaled from the strongest (++++) to the weakest (+).

N. Skin Reactions:

The reactions were based on the method described by Valentine et al. (1967) with a slight modification. The dorsal skin of an anesthetized rat was shaved and skin sites were marked. About 0.05 ml of antiserum of different dilutions was injected intracutaneously into separate sites. Ten to twenty minutes later, the rat was injected intravenously with 0.5 ml of 0.5% Evan's blue in physiological saline. The animal was sacrificed 10 minutes later and the skin was removed for examination. Usually, all the sera to be tested and the controls were injected accordingly into the skin of one animal and a group of three to four rats were used for each test. The results were recorded and averaged. The diameter of each blue spot was measured twice to get the mean value. The color intensity was graded from the strongest (++++) to the weakest (+). Any spot having a mean diameter of less than 5 mm was regarded as a negative result.

IV. RESULTS AND DISCUSSION

A. Properties of Anti-Rat Mast Cell Sera:

Anti-rat mast cell (anti-RMC) sera were induced in three different rabbits as described in Section III:B of this chapter. Sera were collected 7 days after the last injection and were treated as described. The antibody activities of all 3 antisera were screened according to their ability to lyse ^{51}Cr-labelled rat mast cells (RMC) in the presence of rabbit serum complement. The results showed that they were

all cytotoxic to RMC but to different extents (Fig. 1). An antiserum from one of the three rabbits, designated as anti-RMC_3 was more cytotoxic to RMC than the other two. Two of the three antisera, anti-RMC_1 and anti-RMC_3 were used for further studies. It has previously been demonstrated that absorption of anti-RMC sera with rat liver cells could remove some of the non-specific antibodies (Yiu and Froese, 1976). Hence, both anti-RMC sera in the present study were absorbed with rat liver cells five times. It is known that mast cells, at the time of isolation, carry autologous IgE on the surface. As a consequence, it was expected that the anti-RMC sera would contain anti-IgE antibodies. Therefore, the antisera were passed through immunosorbent columns of Sepharose-CL-4B coupled with the immunoglobulin fraction of the ascitic fluid of rats bearing the immunocytoma IR162 (rat IgE myeloma producers). The absorbed anti-RMC sera were then subjected to the following experiments. Both antisera and their unabsorbed counterparts were tested for the presence of anti-IgE antibodies by allowing them to react with ^{125}I-IgE, followed by precipitation of the immune complexes with a goat anti-rabbit immunoglobulin serum (GAR). The amount of GAR used had been predetermined for an optimal precipitation. Both unabsorbed anti-RMC sera precipitated a fair amount of ^{125}I-IgE , especially anti-RMC_1 (Table I), confirming the presence of anti-IgE antibodies. However, after absorption, neither of the two antisera reacted with ^{125}I-IgE indicating the effectiveness of the absorption.

When the antisera were tested for their ability to inhibit ^{125}I-IgE binding to RMC, the absorbed antisera showed different inhibitory effects. Although both anti-RMC_1 and anti-RMC_3 inhibited 70 - 80% of the binding , the absorbed anti-RMC_1 (anti-$RMC_{1,\ abs}$) was less effective

Fig. 1 ^{51}Cr-Release from Rat Mast Cells by Different Anti-RMC Sera

About 0.1 ml of ^{51}Cr-labelled rat mast cells (1 x 10^6 per ml of THM supplemented with 10% FCS) was mixed with 0.1 ml of serum of different dilutions in each well of the microtiter plate. Next, 0.1 ml of fresh rabbit serum (1 : 10) was added into each well. The mixture was incubated at 37°C for 30 mins. The plate was then centrifuged at 200 x g for 8 mins. The supernatant of each well, 0.1 ml in volume, was counted for its radioactive content. After the calculation as, described under METHODS, the results were expressed as % of ^{51}Cr-release.

Anti-RMC$_1$: (●——●) Anti-RMC$_2$: (△——△)

Anti-RMC$_3$: (□——□) NRS : (▲——▲)

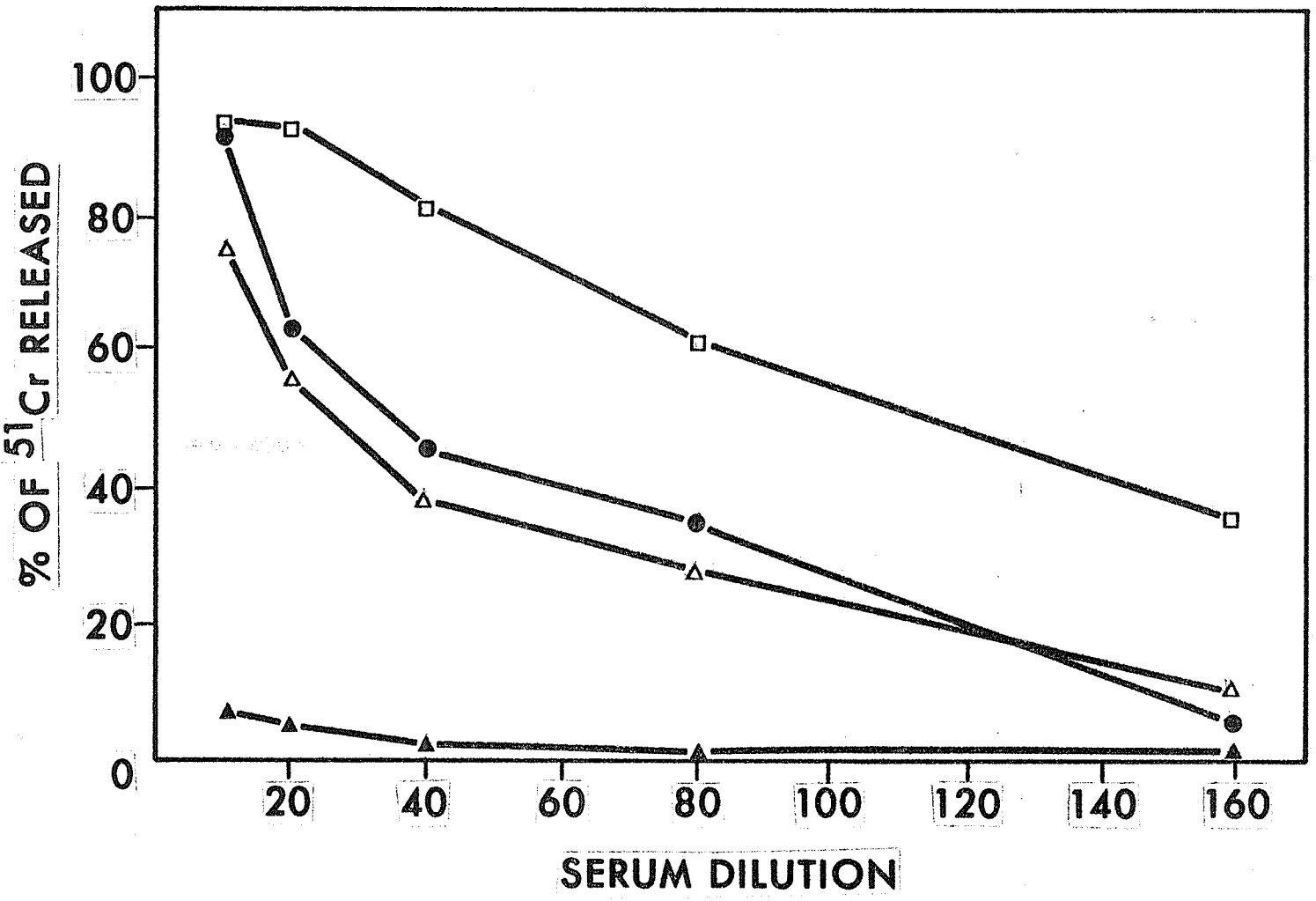

100
80
60
40
20
0
% OF ^{51}Cr RELEASED
20
40
60
80
100
120
140
160
SERUM DILUTION

TABLE I

PRECIPITATION OF ^{125}I-IgE BY DIFFERENT ANTI-RMC SERA[a]

Serum	µg of ^{125}I-IgE Precipitated
Rabbit anti-rat IgE	0.0259
Anti-RMC$_1$	0.0165
Anti-RMC$_{1,\ abs}$	0.0010
Anti-RMC$_3$	0.0100
Anti-RMC$_{3,abs}$	0.0009
NRS$_{abs}$	0.0006

a. About 200 µg of rabbit immunoglobulins from each of the antisera were mixed with 0.05 µg of ^{125}I-IgE followed by the addition of a goat anti-rabbit immunoglobulin serum (0.1 ml). The incubation was carried out at 37°C for a total period of 60 mins and the precipitation took place at 4°C for 3 - 4 hours.

in blocking the binding than the absorbed anti-RMC_3 (anti-$RMC_{3,\ abs}$) Fig. 2). After absorption, anti-$RMC_{3,\ abs}$ could still inhibit 57% of the binding as compared to the value of 9% by anti-$RMC_{1,\ abs}$. In other words, most of the inhibitory activity exhibited by anti-RMC_1 was most likely due to the presence of anti-IgE antibodies.

Surface antigens of RMC associated with the antisera were subjected to the following analysis. The RMC surface was first radioiodinated by the lactoperoxidase catalyzed procedure and subsequently solubilized with PBS/0.5% NP-40 (v/v). The radiolabelled surface extract was then incubated with the appropriate antiserum (0.4 mg of immunoglobulins per sample). The immune complexes were precipitated with a predetermined amount of GAR. The precipitates were dissolved in the buffer containing 2% SDS and urea and were analyzed by SDS-PAGE on 5% gels. The profile of the RMC surface antigens bound by anti-RMC_1 and that by anti-RMC_3 are shown in Fig. 3. Most of the RMC surface antigens that were capable of reacting with either anti-RMC_1 or anti-RMC_3 resided between fraction 15 and fraction 30 with the most predominant peak near fractions 19 and 20. The anti-RMC_1 serum reacted with at least six surface components (Fig. 3a). The profile of surface components associated with anti-RMC_3 (Fig. 3b) was less distinguishable. In addition, anti-RMC_1 seemed to precipitate more surface antigens found between fractions 5 and 12 than anti-RMC_3 did. Analysis of the surface antigens associated with the absorbed antisera revealed two different profiles. Only four components were found to react with anti-$RMC_{1,\ abs}$ (Fig. 3a). The profile of the antigens reacting with anti-$RMC_{3,\ abs}$ was almost identical to that of the antigens reacting with the corresponding unabsorbed serum (Fig. 3b). While

Fig. 2 Inhibition of ^{125}I-IgE Binding to Rat Mast Cells by Different Anti-RMC Sera

Rat mast cells (5×10^5 in 1 ml of THM/BSA) were incubated with 0.4 mg of rabbit immunoglobulins of each antiserum in a silicone grease coated plastic tube at 37°C for 30 mins. At the end of the time, 0.1 µg of ^{125}I-IgE was added to each tube and the incubation was continued for another 30 mins. After thorough washing as described under METHODS, the cell pellets were counted for their radioactive contents. The results were calculated and expressed as % inhibition.

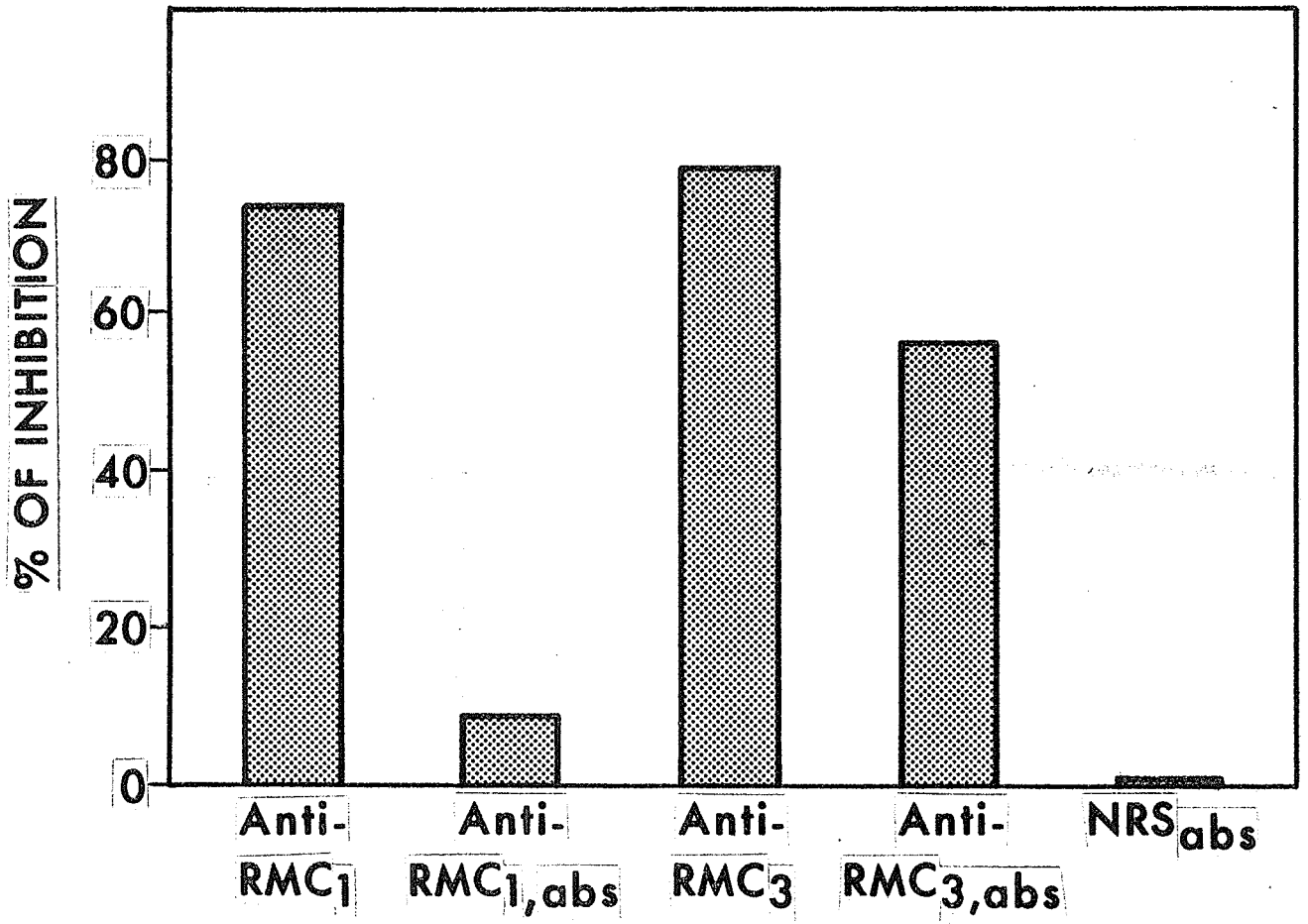
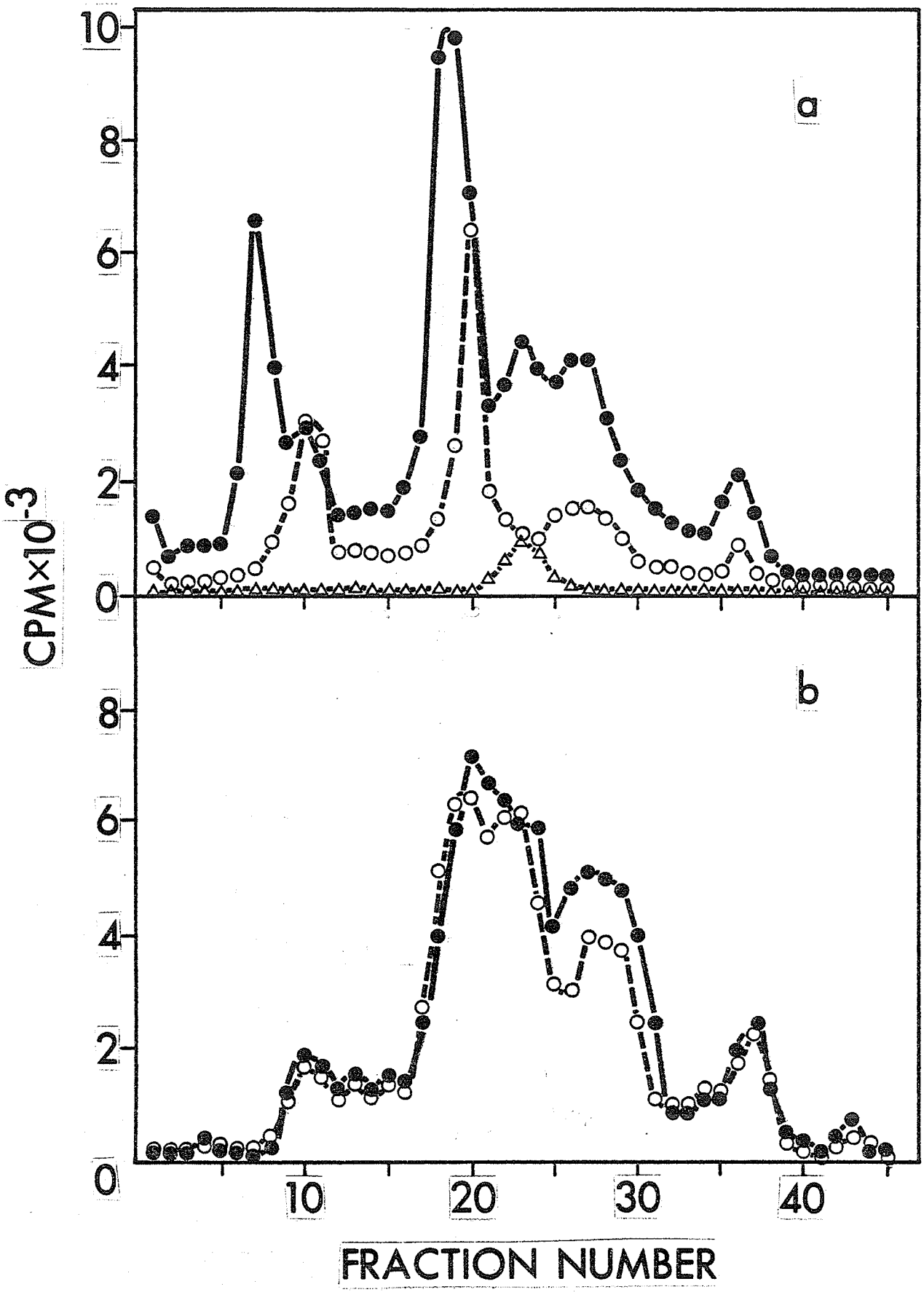

% OF INHIBITION
80
60
40
20
0
Anti-RMC1
Anti-RMC1,abs
Anti-RMC3
Anti-RMC3,abs
NRSabs

Fig. 3 5% SDS-PAGE Analysis of RMC Surface Antigens Precipitated by Different Anti-RMC Sera

Two batches of rat mast cells (5×10^5 per batch) were surface labelled with ^{125}I and solubilized with PBS/0.5% NP-40. After dialysis against PBS/0.5% NP-40 overnight, the pooled cell extract was divided into 4 aliquots each of which was incubated with 0.4 mg of rabbit immunoglobulins of each antiserum at 37°C for 30 mins. A predetermined amount of a goat anti-rabbit immunoglobulin serum was added into each tube and the incubation was continued for another 30 mins. The immunoprecipitates were allowed to form at 4°C for 3 - 4 hours. After thorough washing, the precipitates were dissolved in a sample buffer containing 2% SDS and 9 M urea followed by dialysis and were subsequently analyzed by SDS-PAGE on 5% gels. When anti-IgE was used, 10 µg of IgE was previously added to the cell extract of ^{131}I-labelled RMC for an incubation at 37°C for 30 mins. After the addition of 0.4 mg of the immunoglobulins of goat anti-rat IgE, the precipitates were allowed to form and analyzed as described above.

a. ^{125}I-labelled RMC surface antigens bound by anti-RMC_1 (● ——— ●). ^{125}I-labelled RMC surface antigens bound by anti-RMC_1, abs (o - - - o) were analyzed on the same gel with ^{131}I-labelled RMC surface antigens precipitated by IgE and anti-IgE (Δ ——— Δ).

b. RMC surface antigens bound by : anti-RMC_3 (● ——— ●) and anti-RMC_3, abs (o - - - o)


a
b
CPM×10-3
FRACTION NUMBER
0
2
4
6
8
10
20
30
40

absorption had hardly altered the overall pattern of surface antigens associated with anti-RMC_3, it appeared to have removed antibodies to several antigens from anti-RMC_1. Particularly, antibodies reacting with antigens migrating near fractions 7 and 23 were absorbed. In addition, antibodies reacting with the major component near fraction 18 were removed. The fact that antibodies to several antigens were absorbed from anti-RMC_1 suggests that the absorption may also have been responsible for the drastic reduction in the capacity of anti-$RMC_{1,\ abs}$ to inhibit the binding of IgE to RMC. Some of the eliminated antibodies may have reacted with antigens in close proximity to the receptor for IgE.

In order to establish the presence of anti-receptor antibodies in the anti-RMC sera, the following experiment was performed. One batch of RMC was surface-labelled with ^{125}I followed by solubilization with PBS/NP-40. A portion of this surface extract was allowed to react with anti-$RMC_{1,\ abs}$ and an appropriate amount of GAR was added to the mixture to form immunoprecipitates. Another batch of cells was surface-labelled with ^{131}I. A portion of the cell extract was incubated with 10 μg of IgE and the receptor-IgE complexes were precipitated with goat anti-rat IgE. Both precipitates were dissolved in the SDS-urea containing buffer, mixed and were analyzed by SDS-PAGE on the same 5% gel. The result (Fig. 3a) showed that the ^{131}I-labelled receptor component appeared around fraction 23 while none of the surface antigens precipitated by anti-$RMC_{1,\ abs}$ was found in this fraction. By contrast, anti-$RMC_{3,\ abs}$ reacted with a RMC surface component which exhibited a peak near fraction 23. Even though the presence of a component in this fraction did not prove that it was the receptor, it was,

nevertheless, concluded that the likelihood of finding anti-receptor antibodies in anti-$RMC_{3,\ abs}$ was greater than finding them in anti-$RMC_{1,\ abs}$. Indeed, any presence of anti-receptor antibodies in anti-$RMC_{3,\ abs}$ and not anti-$RMC_{1,\ abs}$ could provide a further explanation of why the former antiserum was much more effective in blocking the binding of IgE to RMC.

In view of the fact that anti-$RMC_{3,\ abs}$ reacted with more surface antigens than anti-$RMC_{1,\ abs}$ and that it showed a greater promise of containing anti-receptor antibodies, this serum was used for all further studies. Henceforth, it will simply be referred to as anti-RMC.

B. Properties of Anti-Rat Basophilic Leukemia Cell Sera:

Anti-rat basophilic leukemia (anti-RBL) cell sera were induced in three rabbits and the antisera were obtained and treated as mentioned before. The antibody activity of these sera was screened by the assay of cytotoxic ^{51}Cr-release. The results showed that all the antisera were cytotoxic to rat basophilic leukemia (RBL) cells in the presence of guinea pig complement (Fig. 4). Thus, anti-RBL_1 was the most potent antiserum, causing 50% ^{51}Cr-release at a dilution of 1 : 60, whereas anti-RBL_2 and anti-RBL_3 were less potent and would only bring about the same effect at dilutions of 1 : 40 and 1 : 30, respectively.

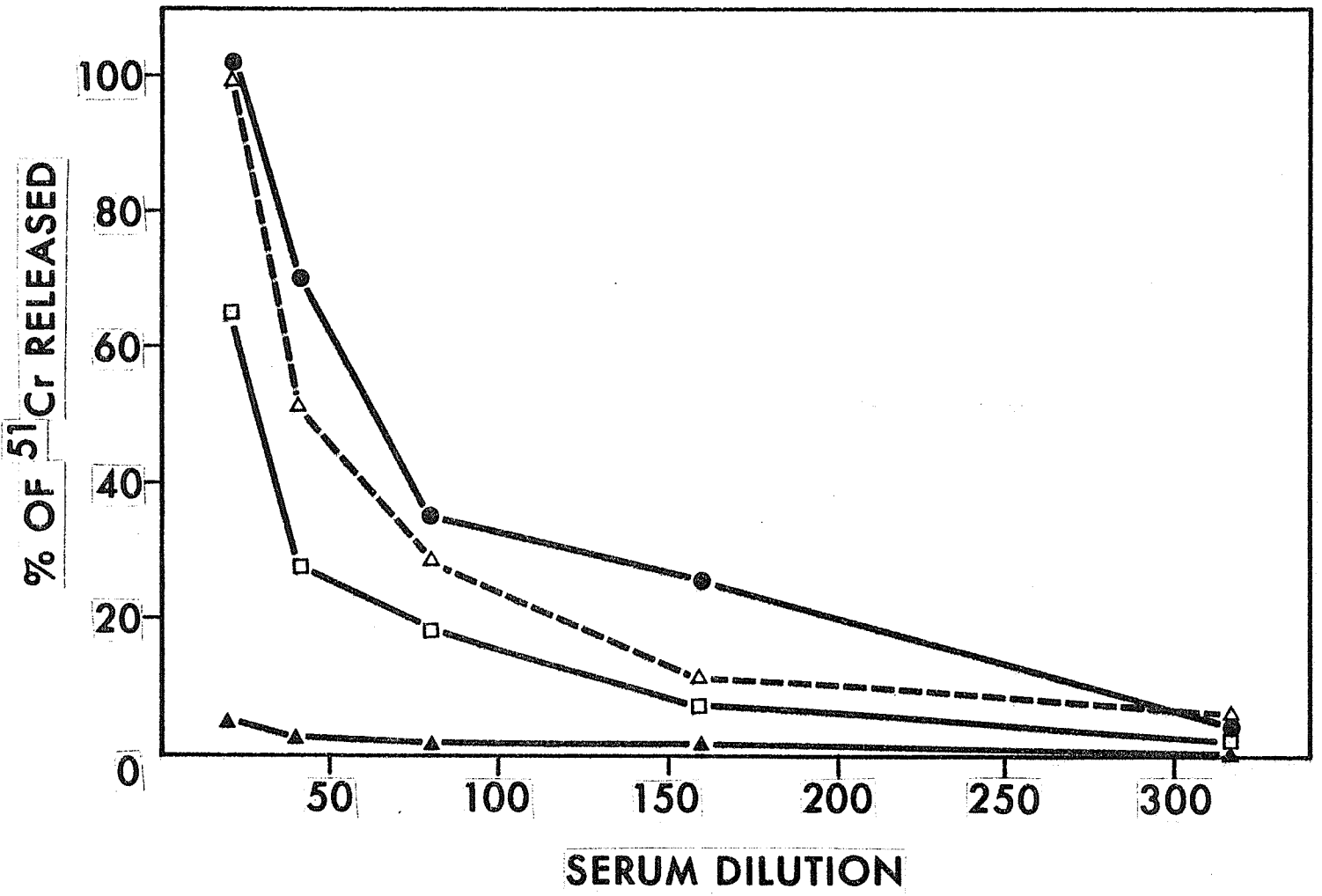
The antisera were then tested for their relative capacity to react with RBL cell surface components by the following experiment. A batch of RBL cells (3×10^7) was first surface-labelled with 0.9 mCi of ^{125}I followed by detergent extraction. The cell extract was divided into four aliquots, each of which was incubated with one of the antisera or normal rabbit immunoglobulins. The amount of rabbit immunoglobulins used for the reaction was kept constant for all four and it

Fig. 4 ^{51}Cr-Release from Rat Basophilic Leukemia Cells by Various Anti-RBL Cell Sera

^{51}Cr-labelled rat basophilic leukemia cells (1×10^5) were mixed with 0.1 ml of antiserum of different dilutions in each well of the microtiter plate. After the addition of 0.1 ml of guinea pig complement per sample, the mixture was incubated at 37°C for 30 mins. The plate was then centrifuged at 200 x g for 8 mins. The supernatant of each well, 0.1 ml in volume, was counted for its radioactive content. The results were calculated and expressed as % of ^{51}Cr released.

Anti-RBL$_1$: (●————●) Anti-RBL$_2$: (△————△)

Anti-RBL$_3$: (□————□) NRS : (▲————▲)


100
80
60
40
20
0
% OF 51Cr RELEASED
50
100
150
200
250
300
SERUM DILUTION

was equivalent to a protein concentration of 0.4 mg per sample. The immune complexes were then insolubilized with Protein A-Sepharose. After thorough washing with PBS/0.1% NP-40 five times, the bound radioactivity of Protein A-Sepharose was determined. At the same concentration, anti-RBL_1 precipitated about 2% of the total extract added whereas the values for anti-RBL_2 and anti-RBL_3 were 1.7% and 1.5%, respectively. The amount of added RBL cell extract precipitated by normal rabbit serum was even less (Table II), indicating that the antisera did react with some of the RBL cell surface antigens.

Next, RBL cell surface antigens bound by the three antisera were subjected to SDS-PAGE analysis. The cells were once again surface-labelled followed by solubilization with PBS/NP-40. The surface antigens were then isolated by means of the three anti-RBL sera in the presence of Protein A-Sepharose. The immunoglobulins of a normal rabbit serum served as a control (NRS). Bound material was eluted as described and analyzed by SDS-PAGE on 10% gels. The results showed that most of the RBL cell surface antigens reacting with any of the three antisera migrated between fractions 7 and 50 (Fig. 5), whereas none of them seemed to react with NRS. All three antisera yielded very similar patterns of 8 - 10 components with different mobilities. The peak near fraction 68 (Fig. 5a) most likely consisted of ^{125}I-labelled lipids which migrated with the tracking dye. It is interesting to note that all three antisera yielded almost identical patterns and had a very similar capacity to precipitate the surface-labelled antigens (Table II). This is in contrast to the anti-RMC sera, each of which reacted with the RMC surface antigens quite differently and as a consequence yielded varying SDS-PAGE patterns (Fig. 4). The reason for the rather

TABLE II

AMOUNT OF RBL CELL SURFACE ANTIGENS PRECIPITATED BY DIFFERENT ANTI-RBL CELL SERA[a]

Serum	cpm of Cell Extract Added	cpm of Cell Extract Bound	% Bound
Anti-RBL_1	1.5×10^7	3.0×10^5	2.00
Anti-RBL_2	1.5×10^7	2.6×10^5	1.70
Anti-RBL_3	1.5×10^7	2.3×10^5	1.50
NRS	1.5×10^7	6.3×10^4	0.42

a. A batch of rat basophilic leukemia cells (3×10^7) was first surface-labelled with 0.9 mCi of ^{125}I followed by solubilization with PBS/0.1% NP-40. The cell extract was divided into 4 aliquots, each of which was incubated with 0.4 mg of rabbit immunoglobulins of each antiserum or normal rabbit serum (NRS) at 37°C for 30 mins. The immune complexes were then insolubilized with Protein A-Sepharose. After thorough washing with PBS/0.1% NP-40, the bound radioactivity of each sample was counted.

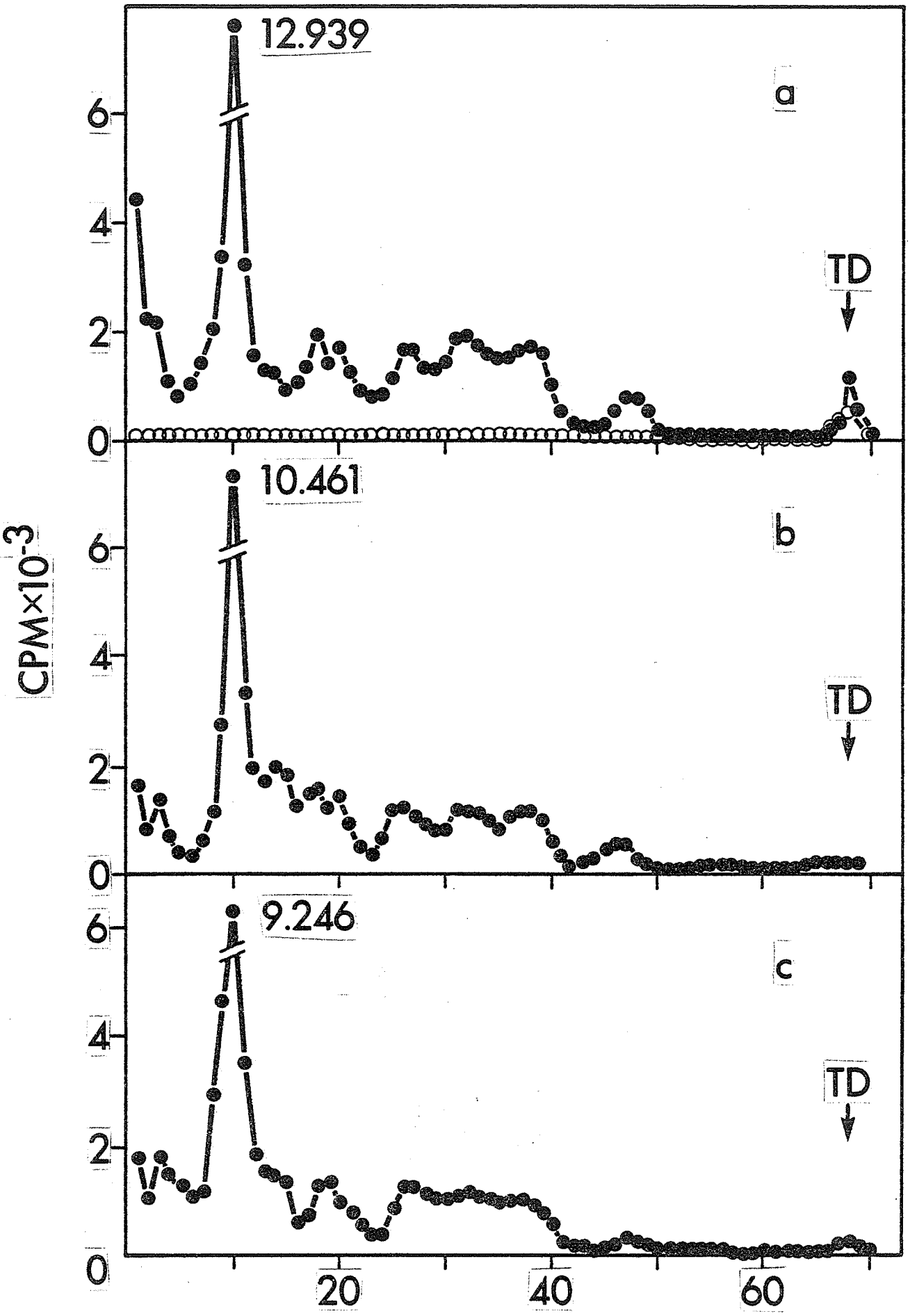
Fig. 5 10% SDS-PAGE Analysis of RBL Cell Surface Antigens Precipitated by Different Anti-RBL Cell Sera

Two batches of RBL cells (2×10^7 per batch) were surface labelled and solubilized as described under METHODS. After dialysis against PBS/0.1% NP-40, the pooled cell extract was redivided into 4 aliquots, each of which was incubated with 0.4 mg of immunoglobulins of the different rabbit sera at 37°C for 30 mins. The immune complexes were insolubilized by Protein A-Sepharose and were subsequently analyzed by SDS-PAGE on 10% gels.

(a) RBL cell surface antigens precipitated by :
Anti-RBL_1 (●————●) and NRS (○————○)

(b) RBL cell surface antigens bound by Anti-RBL_2

(c) RBL cell surface antigens bound by Anti-RBL_3


12.939
a
TD
10.461
b
TD
9.246
c
TD
CPM×10-3
0
2
4
6
20
40
60
FRACTION NUMBER

similar patterns of antigens reacted with the anti-RBL sera can most likely be attributed to the fact that all three rabbits were immunized with RBL cells from the same culture pool and that these cells are homogeneous, exhibiting the same surface antigens from time to time. On the other hand, RMC used for the immunization had to be pooled from several rats which were not inbred and all three rabbits were injected with different RMC pools at different times.

The anti-RBL_1 serum was chosen for further studies because: (1) it was the most cytotoxic antiserum of the three, (2) the antigens which it precipitated yielded a well defined SDS-PAGE pattern and (3) it had a slightly greater capacity to precipitate RBL cell surface antigens than the other two antisera. It will be referred to as anti-RBL from now on.

C. Characterization of the Cross-Reacting Anti-RMC and Anti-RBL Cell Sera:

Rat mast cells used in the present study came from the peritoneal cavity of Lewis rats while RBL cells originated from tumors in Wistar rats. Therefore, in an attempt to render the antisera specific for either RMC or RBL cells and hence, to remove antibodies to antigens common to other Lewis and Wistar cells, both antisera were absorbed with liver cells from Lewis rats three times followed by two absorptions with liver cells from Wistar rats. The anti-RMC serum was further absorbed with immunosorbents containing the immunoglobulin fraction of the ascitic fluid IR162 which contains an IgE myeloma protein. The absorbed antisera will be referred to as anti-RMC_{abs} and anti-RBL_{abs}, respectively. Some properties of anti-RMC absorbed with liver cells of Lewis rats were already described in Section IV:A of

this chapter.

In order to find out if the above absorptions could effectively eliminate some of the non-specific antibodies present in the antisera, these sera were tested for their cytotoxic activity to lymph node (LN) cells, the surface of which contains most of the common rat surface antigens. Both anti-RMC and anti-RBL were cytotoxic to LN cells in the presence of guinea pig complement, but absorption eliminated most of their cytotoxic activity (Fig. 6), indicating that the absorptions had been effective. Next, the antisera were screened for their anti-IgE activities. The anti-RMC serum which had been shown to contain anti-IgE antibodies before was used as one of the controls for the test. Results from the ^{125}I-IgE-precipitation test showed that anti-RBL contained very little anti-IgE activity. This had to be expected since cultured RBL cells, which were used for the immunization, do not carry IgE on their surface. By contrast, anti-RMC had anti-IgE activity which could be absorbed with the IgE-containing immunoglobulin preparation (Table III).

Both antisera had been shown previously to be cytotoxic to their own target cells. Their cross-reactivities were investigated in the following experiments. First, both types of antisera were tested for their cytotoxicity to RMC and RBL cells separately. Both anti-RMC and anti-RBL could lyse both types of cells in the presence of serum complement (Fig. 7). In addition, both of the absorbed antisera were also cytotoxic to the two target cells. The cytotoxicity was shown to be specific since neither normal rabbit serum nor an absorbed anti-rat sarcoma cell serum (anti-S_{abs}) could kill RMC or RBL cells under the same condition. It should be noted that the cytotoxic activity of each

Fig. 6 Cytotoxicity of Various Rabbit Antisera for Rat Lymph Node Cells

Rat lymph node cells (1×10^7) were labelled with 0.1 mCi of $Na_2(^{51}Cr)O_4$ at 37°C for 30 minutes followed by thorough washing as described under METHODS. The cells were readjusted to 1×10^6 per ml of THM supplemented with 10% FCS. About 1×10^5 cells were mixed with 0.1 ml of rabbit serum of different dilutions in each well of the microtiter plate. After an addition of 0.1 ml of guinea pig complement (1 : 10) to each sample, the plate was incubated at 37°C for 30 mins. At the end of the time, the plate was centrifuged at 200 x g for 8 mins. The supernatant (0.1 ml) of each well was carefully removed and counted for its radioactive content. The results were calculated and expressed as % ^{51}Cr release.

Anti-RMC : (▲——▲) Anti-RMC$_{abs}$: (Δ——Δ)

Anti-RBL : (●——●) Anti-RBL$_{abs}$: (○——○)

NRS : (□——□)

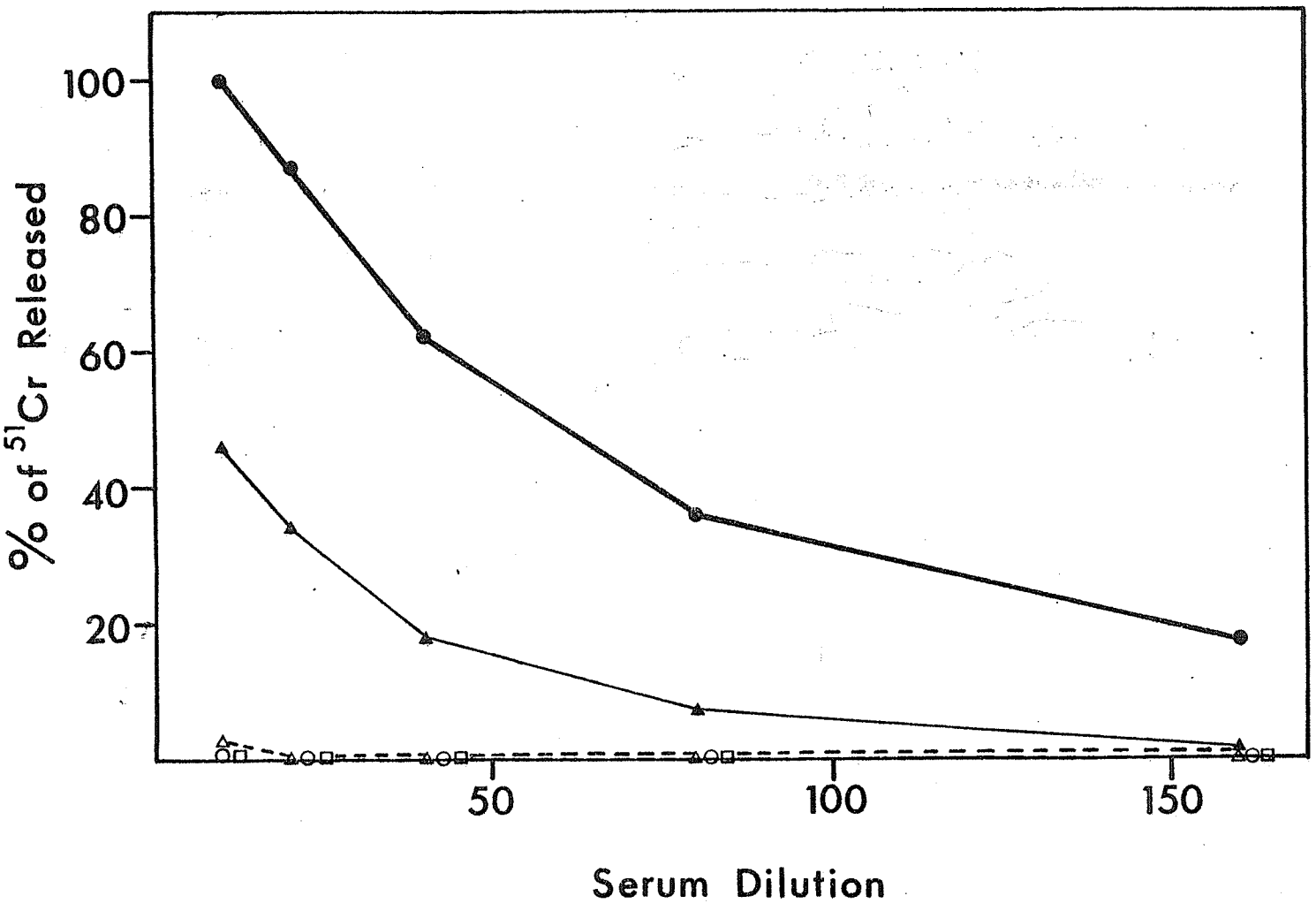

100
80
60
40
20
% of ^{51}Cr Released
50
100
150
Serum Dilution

TABLE III

*PRECIPITATION OF ^{125}I-IgE BY ANTI-RMC AND ANTI-RBL CELL SERA

Serum	µg of ^{125}I-IgE Precipitated
Rabbit anti-rat IgE	0.0318
Anti-RMC	0.0088
Anti-RMC$_{abs}$	0.0001
Anti-RBL	0.0004
NRS$_{abs}$	0.0000

* The experimental condition was similar to that described in the footnote of Table I. Both anti-RMC$_{abs}$ in this table and anti-RMC$_{3,\ abs}$ in Table I came from the same antibody preparation.

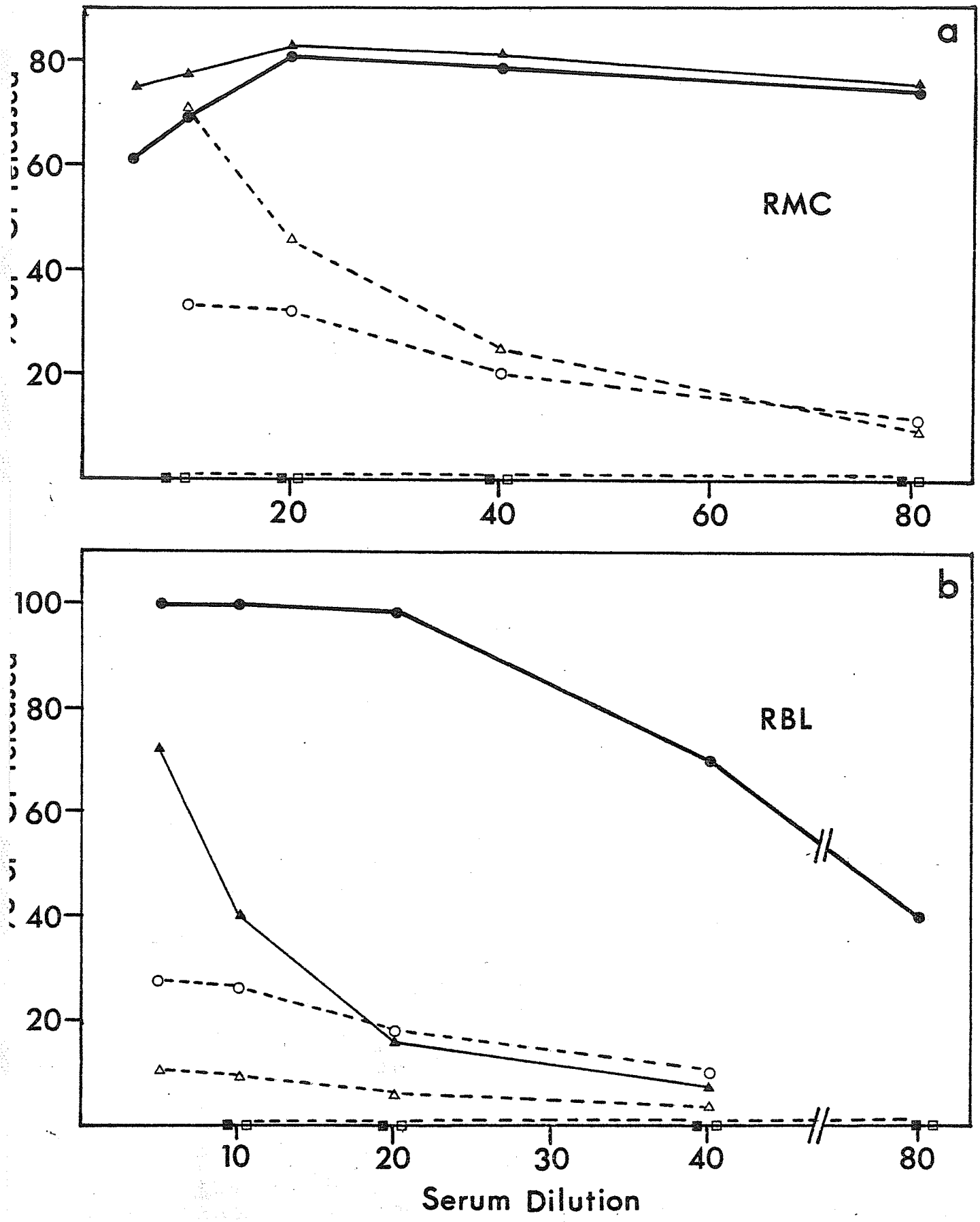
Fig. 7 Cytotoxicity of Various Rabbit Antisera to RMC and RBL Cells

Experimental conditions : see the legends of Figs. 1 & 4 for ^{51}Cr-release from (a) RMC and (b) RBL cells, respectively.

Anti-RMC : (▲———▲) Anti-RMC$_{abs}$: (△———△)

Anti-RBL : (●———●) Anti-RBL$_{abs}$: (○———○)

Anti-S$_{abs}$: (■———■) NRS : (□———□)


a
80
60
40
20
RMC
20
40
60
80
b
100
80
60
40
20
RBL
10
20
30
40
80
Serum Dilution

antiserum to the cells against which it was raised (anti-RMC or anti-RMC_{abs} versus RMC; anti-RBL or anti-RBL_{abs} versus RBL cells) was much stronger than its cross-reactivity (e.g., anti-RMC_{abs} versus RBL cells; anti-RBL_{abs} versus RMC). However, when RMC were used as target cells, this difference between the antisera became only noticeable after absorption (Fig. 7a). This suggests that anti-RBL probably contained a larger number of antibodies directed against antigens common to several types of rat cells than anti-RMC.

Binding between antibodies of the absorbed antisera and the membrane surface of RMC and RBL cells was examined by indirect immunofluorescence. The cells were incubated with the appropriate antiserum and a fluoresceinated goat anti-rabbit immunoglobulin serum (FITC-GAR), as described in Section III:M of this chapter. While neither normal rabbit serum, which had been pre-absorbed with rat erythrocytes (NRS_{abs}), in combination with FITC-GAR nor FITC-GAR alone could stain the cells, both of the absorbed antisera caused staining of the two cell types. However, the intensity of fluorescence varied in each case (Table IV). The anti-RMC_{abs} serum reacted more strongly with RMC than with RBL cells. A photograph was taken to show the weak fluorescence intensity of RBL cell surface stained by anti-RMC_{abs} (Fig. 8). A high intensity of fluorescence could be observed around the circumference of RBL cell surface when anti-RBL_{abs} (1 : 20) was present. The staining was found evenly distributed around the circumference of the cells. On the contrary, the staining of the RMC surface by anti-RBL_{abs} was relatively weak. The specificity of staining was further verified by using rat peritoneal cells which contained mostly lymphocytes, macrophages and 10% RMC. About 100 cells were examined

TABLE IV

INDIRECT IMMUNOFLUORESCENCE[a]

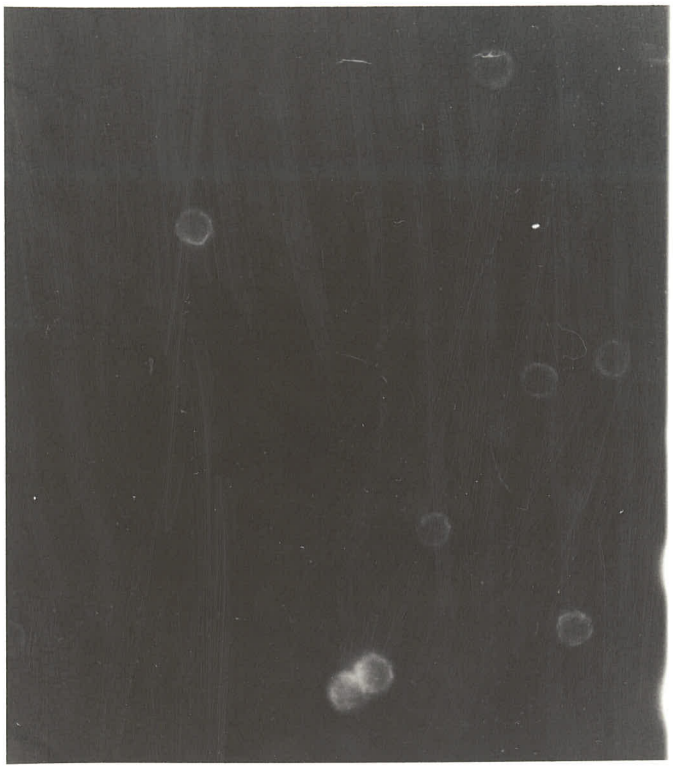
Cell Type	Antiserum	Dilution	Fluorescence Intensity
RMC	Anti-RMC$_{abs}$	1 : 20	++++
		1 : 40	+++
	Anti-RBL$_{abs}$	1 : 20	++
		1 : 40	+
	NRS$_{abs}$	1 : 10	-
	[b]	-	-
RBL Cells	Anti-RMC$_{abs}$	1 : 20	++
		1 : 40	+
	Anti-RBL$_{abs}$	1 : 20	++++
		1 : 40	+++
	NRS$_{abs}$	1 : 10	-
	[b]	-	-

a Cells (1 x 10^5) in 0.1 ml of medium were incubated with an equal volume of diluted antiserum or NRS$_{abs}$ at 4°C for 30 mins. After washing, they were resuspended in 0.1 ml of medium and were incubated with 0.1 ml of FITC-GAR (1 : 10) for another 30 mins. Intensity was recorded from the strongest (++++) to the weakest (+) or as negative (-).

b Cells were incubated with FITC-GAR alone.

Fig. 8 Indirect Immunofluorescent Staining of Rat Basophilic Leukemia Cells

RBL cells (1×10^5) in 0.1 ml of THM/BSA containing 10 mM of sodium azide were incubated with 0.1 ml of anti-RMC_{abs} (1 : 20) at $4^{\circ}C$ for 30 mins. The cells were then washed with the medium by centrifugation for at least three times and were suspended in the same medium. After the addition of 0.1 ml of FITC-GAR (1 : 10), the incubation was continued for another 30 mins. After thorough washing, the cells were finally suspended in 0.1 ml of the same medium containing 20% glycerol as an addition. A drop of the cell suspension was placed on a glass slide with a cover slip sealed with nail vanish. The cells were examined as described under METHODS. The photograph was taken with a Leitz camera attached to the microscope. The film used was Kodak B/W of 400 ASA.



each time. It was found that anti-RMC_{abs} could stain about 25% of all other cells besides RMC, although the staining intensity was weaker in the former than in the latter. This finding indicated that previous absorptions with rat liver cells only removed most of the cytotoxic antibodies to lymph node cells. Other non-specific antibodies, especially those that could be directed against common surface antigens of the peritoneal cells might still be present in the anti-RMC_{abs} serum. Hence, mast cell-depleted peritoneal cells were used for the absorptions in addition to those with liver cells for some aliquots of anti-RMC. When the latter were tested for their staining ability on peritoneal cells other than RMC, it was found that the number of non-specifically stained cells had decreased considerably but not completely. The fact that anti-RMC_{abs} weakly stained non-RMC peritoneal cells is somewhat in contrast to the observation that the same serum was no longer cytotoxic to lymph node cells. Several suggestions can be invoked to explain these observations: (1) the non-RMC peritoneal cells carried antigens different from those of lymph node or liver cells, (2) the fluorescence staining technique is more sensitive than the cytotoxic ^{51}Cr-release method, or (3) the absorption had removed all cytotoxic antibodies but had failed to eliminate some of the non-cytotoxic ones. Although absorption with mast cell-depleted peritoneal cells had decreased the staining of non-RMC population, it also weakened the staining intensity of RMC by anti-RMC_{abs}. This finding suggests that some anti-RMC antibodies may cross-react weakly with some non-RMC cells of the rat peritoneum. The above absorption had no effect on the staining of RBL cells by anti-RMC_{abs}. On the other hand, anti-RBL_{abs} did not stain any cells

other than mast cells when the peritoneal cells were used. Furthermore, a second anti-RMC serum, anti-$RMC_{1,\ abs}$ mentioned in Section IV:A of this chapter, stained both RMC and other peritoneal cells but failed to stain any RBL cells. The above findings indicate that (1) even though antibodies against some common surface antigens of peritoneal cells were still present in the anti-RMC_{abs} serum, they did not cross-react with RBL cells and (2) the choice of using anti-$RMC_{3,\ abs}$ instead of anti-$RMC_{1,\ abs}$ for the study was correct since the former reacted with RBL cells.

The cytotoxic activities of anti-RMC and anti-RBL cell sera towards lymph node cells demonstrated that both antisera contained non-specific antibodies against antigens that were common between liver cells, lymph node cells, RMC and RBL cells. Absorption of the antisera with liver cells did remove such antibodies. The anti-RBL cell serum seemed to contain mostly RBL cell specific antibodies and possibly some cross-reacting antibodies to RMC after the absorptions. On the other hand, the anti-RMC serum contained antibodies against the cytophilic rat IgE immunoglobulin as well as rat common peritoneal cell surface antigens in addition to mast cell-specific and possibly some cross-reacting antibodies to RBL cells. Appropriate absorptions could considerably eliminate antibodies of the former two from the anti-RMC serum. The possible cross-reactivity of both anti-RMC_{abs} and anti-RBL_{abs} would suggest the presence of antibodies against surface antigens shared between RMC and RBL cells. These antigens were absent from the surface of lymph node and liver cells as well as other non-RMC peritoneal cells which are mainly lymphocytes and macrophages. Hence, the nature of the cross-reacting antibodies in anti-RMC and

anti-RBL cell sera, will be investigated by the following studies.

Valentine et al. (1967) first reported on the ability of an anti-RMC serum to induce an immediate anaphylactic skin reaction in normal rats. Thus, the effect of both anti-RMC_{abs} and anti-RBL_{abs} on inducing the skin reaction was investigated. The results showed that while neither of the rat erythrocyte-absorbed normal rabbit serum (NRS_{abs}) nor PBS could induce any positive skin reaction, both antisera elicited blue spots of different diameters and color intensities depending on their serum dilutions (Table V). An example of a typical skin reaction of this kind is illustrated by a photograph taken from the author's previous study (M.Sc. Thesis, 1976) (Fig. 9). While anti-RMC_{abs} was capable of inducing a positive skin reaction even at a dilution of 1 : 200, anti-RBL_{abs} had the same capability except that the spots that it elicited were smaller and not as strong in terms of color intensity. The results were as expected since the cross-reacting antibodies were not necessarily as potent and as numerous as the specific antibodies. This skin reaction is caused by the release of mediators of immediate hypersensitivity from skin mast cells of the rat. The binding of the antibodies to the surface of these cells probably triggered the reaction which was in the form of a cytotoxic release. The latter statement is based on the author's previous finding that anti-RMC_{abs} could only induce the degranulation of RMC in the presence of serum complement (M.Sc. Thesis, 1976).

The nature of the cross-reacting antibodies was further revealed by the following studies. Antisera were used to inhibit IgE binding to either RMC or RBL cells. Both anti-RMC and anti-RBL alone blocked the binding between IgE and either cell type (Fig. 10). This inhibi-

TABLE V

SKIN REACTIONS INDUCED BY ANTI-RMC AND ANTI-RBL CELL SERA*

Dilution / Serum	Diameter of Spot (mm)			Color Intensity
	1 : 10	1 : 100	1 : 200	
$Anti\text{-}RMC_{abs}$	13.5	7.5	6	++++
$Anti\text{-}RBL_{abs}$	10.0	6.0	5	+++
NRS_{abs}	-	-		-
PBS				-

* About 0.05 ml of antiserum of different dilutions were injected into the rat skin intracutaneously. The rat was challenged 10 to 20 mins later and was sacrificed 10 mins later. Results were recorded in terms of the diameter of the spot and its color intensity which graded from the strongest (++++) to the weakest (+) or as negative (-). Any spot having a diameter of less than 5 mm was regarded as negative. The above results were the mean values obtained from three rats.

Fig. 9 A Typical Example of the Skin Reaction

Experimental conditions : see the footnote to Table V.

Spot A : It represents a positive skin reaction with the strongest color intensity (++++).

Spot B : A negative reaction induced by PBS.

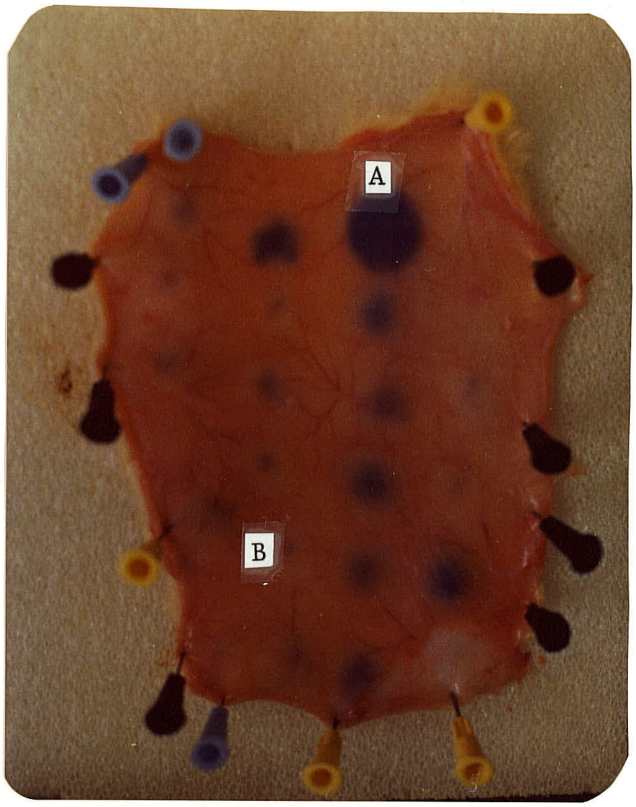
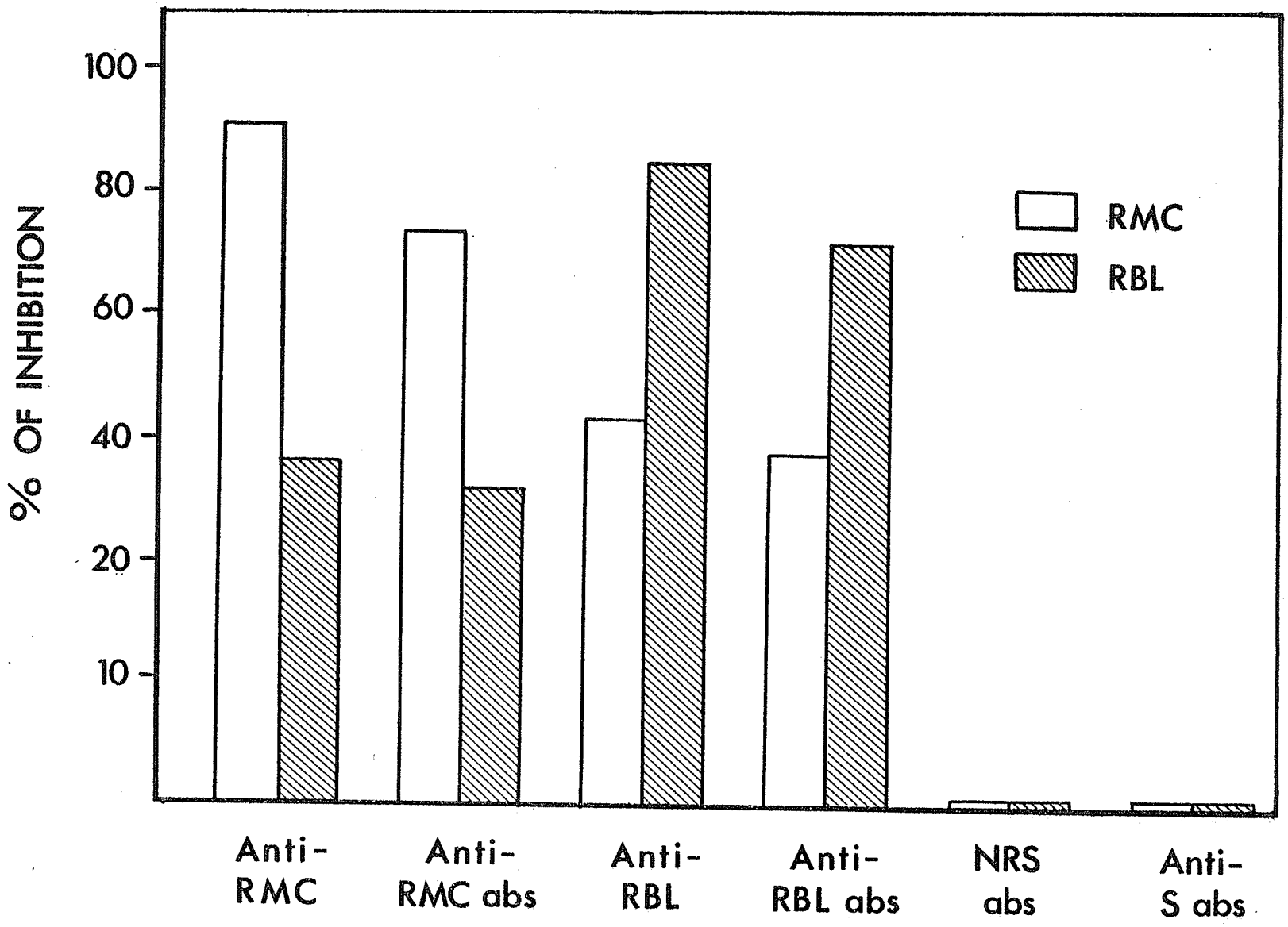

A
B

Fig. 10 Inhibition of IgE Binding to RMC and RBL Cells by Different Antisera

About 0.4 mg of rabbit immunoglobulin of each antiserum were incubated with 1 x 10^6 RBL cells or RMC at 37°C for 30 mins. The incubation was continued for another 30 mins after the addition of 0.1 µg of ^{125}I-IgE to each sample. After thorough washing, the cell pellets were counted for their radioactive contents and the results were calculated and expressed as % inhibition.


% OF INHIBITION
100
80
60
40
20
10
RMC
RBL
Anti-
RMC
Anti-
RMC abs
Anti-
RBL
Anti-
RBL abs
NRS
abs
Anti-
S abs

tory activity alone was not sufficient to provide evidence of specificity for the receptor for IgE since it had been shown that even a rabbit anti-rat sarcoma cell serum could block the binding under similar conditions (Yiu and Froese, 1976). Absorptions with liver cells could abolish the blocking effect of this antiserum which had been referred to as the anti-S_{abs} serum in that text. Normal rabbit serum which had been pre-absorbed with rat erythrocytes had no effect on the binding either. On the other hand, absorptions did not abolish the capacity of both anti-RMC and anti-RBL to inhibit IgE binding to either cell type. The slight decrease in the blocking activity of both anti-RMC_{abs} and anti-RBL_{abs} was probably due to the dilution effect resulting from numerous absorption procedures. The discriminating effects which had been observed in results obtained from the cytotoxicity test (Fig. 7) and the immunofluorescent staining (Table IV) also existed in the data obtained from this study (Fig. 10). The anti-RMC sera blocked the IgE-binding to RMC more effectively than they blocked the binding to RBL cells. The anti-RBL serum also showed a more pronounced effect on its own, original target cells.

Based on the above findings, it became clear that cross-reacting antibodies were present in both of the anti-RMC_{abs} and anti-RBL_{abs} sera. These antibodies were responsible for the abilities of the antisera to: (1) bind to the surface of RMC and RBL cells (indirect immunofluorescence), (2) elicit immediate anaphylactic skin reactions and (3) inhibit the binding of IgE to both types of cells. The results suggest that both RMC and RBL cells might share certain common surface antigens that are absent on some other, or at least liver and lymph node, cells. Since the binding between the cross-reacting antibodies and

these surface antigens could effectively block the accessibility of the receptors to IgE molecules, the inhibition could be due to the following situations. These antibodies might bind to surface antigens that are near the receptor, thereby blocking the IgE binding by mere steric hindrance. Alternatively, these antibodies could inhibit the binding by directly reacting with the antigenic determinant present on the IgE binding site in the membrane of RMC and RBL cells.

The results reported so far have clearly demonstrated that both anti-RBL_{abs} and anti-RMC_{abs} reacted quite well with RMC and RBL cells, respectively. However, the reactivity with the homologous cell type was always more pronounced. These results suggested that the two antisera contained antibodies to common antigens as well as antigens specific to either cell type. In addition, the fact that either antiserum inhibited the binding of IgE to either RMC or RBL cells raised the possibility that antibodies to the receptors for IgE were present in both antisera and that, as could be expected, this receptor represented an antigen common to both cell types. Investigation aimed at more clearly demonstrating these possibilities is presented in the next chapter.

CHAPTER III

CHARACTERIZATION OF THE RMC AND RBL CELL SURFACE ANTIGENS

I. INTRODUCTION

Various anti-rat mast cell (anti-RMC) and anti-rat basophilic leukemia (anti-RBL) cell sera have been induced in the past. Most of these were used mainly to study the functional aspects of rat mast cells or to characterize the receptor for IgE on the RBL cell surface (Chapter I, Section VI). Only one antiserum to whole cells (RMC) has so far been used to investigate the surface antigens of RMC (Yiu and Froese, 1976). One of the initial aims of this study was to clearly demonstrate the presence of antibodies to the receptor for IgE in antisera to whole RMC and RBL cells. One previous study (Yiu and Froese, 1976) had shown that an antiserum to RMC most likely contained antibodies to the receptor for IgE on these cells. Yet, it did not prove it beyond any doubt. While the present study was in progress, reports appeared indicating that such antibodies were present in anti-RBL cell sera. Also, several antisera to purified receptors of RBL cells were produced and it was shown that such antibodies cross-reacted with the receptor for IgE present on the RMC surface (Chapter I, Section III: A & B). Therefore, it was decided to extend the scope of the present investigation and to delineate those antigens which are common to both cell types and those that are specific to each. The characterization

of such antigens may eventually help to elucidate the mechanism whereby cross-linking of receptors for IgE by antibodies (Isersky et al., 1978; Ishizaka et al., 1977b) can induce the same effect of mediator release as the bridging of other surface molecules of mast cells by other antibodies (Daëron and Voisin, 1978 & 1979; Mossmann et al., 1976, 1978 & 1979). In addition, antisera to common and specific antigens of both RMC and RBL cells may be useful for studying the origins of these cells. It was expected that the receptor(s) for IgE would be among these antigens.

II. MATERIALS

They are the same as in Chapter II, Section II.

III. METHODS

A. SDS-PAGE Analysis of Surface Antigens:

The techniques are basically the same as the ones described in Chapter II, Section III:J, K & L. Unless stated specifically therwise, most of the materials were analyzed under non-reducing conditions.

B. Binding of ^{125}I-FCS:

Heat inactivated fetal calf serum (FCS) was radioiodinated by the same chloramine T procedure as described in Chapter II, Section III:G. Fetal calf serum was diluted to an appropriate concentration with PBS and the optical density of the solution was measured with a spectrometer at 280 nm. Its protein concentration was determined by using an extinction coefficient of 7.0 ($E^{1\%}_{280\ nm}$) (William and Chase, 1968). About 0.1 mg of FCS was labelled with 0.35 mCi of

carrier-free ^{125}I. After dialysis against PBS at 4°C overnight, the solution containing ^{125}I-FCS was further diluted to a concentration of 1 µg of ^{125}I-FCS per ml. All aliquots at such a concentration were stored at -70°C for future use.

1. Binding of ^{125}I-FCS by various antisera

Aliquots of ^{125}I-FCS were incubated with different antisera at a concentration of 1 µg of ^{125}I-FCS for every 0.4 mg of rabbit immunoglobulin tested. The incubation was carried out at 37°C for 30 minutes. The antigen-antibody complexes were insolubilized by adding 0.1 ml of Protein A-Sepharose (Pharmacia, Uppsala, Sweden) to each sample. After constant shaking at room temperature for 90 minutes, the mixture was centrifuged at 1000 rpm for 8 minutes. The supernatant was discarded and the absorbent was washed with PBS by centrifugation at least five times. The radioactive content of the samples were determined by counting each tube containing Protein A-Sepharose. Results were expressed as µg of ^{125}I-FCS bound which was calculated as follows:

$$\text{µg of } {}^{125}\text{I-FCS bound} = \frac{\text{cpm bound materials}}{\text{cpm 1 µg of } {}^{125}\text{I-FCS}}$$

2. Binding between ^{125}I-FCS and RBL cells

The cells were adjusted to 3×10^7 per ml and were washed with THM buffer twice. The cell pellet was resuspended in the same buffer and the suspension was divided into three aliquots each containing 1×10^7 cells in a volume of 1 ml of THM. Each aliquot was incubated with 1 µg of ^{125}I-FCS at different temperatures for 1 hour. The cells were washed with THM by centrifugation at 200 x g for 5 minutes. The washing procedure was repeated five times. The cell pellets were then counted for their radioactive contents. Results were expressed

as a percentage of ^{125}I-FCS bound which was calculated from the following formula:

$$\% \text{ of } ^{125}\text{I-FCS bound} = \frac{\text{cpm of cell pellet}}{\text{cpm of 1 } \mu\text{g of } ^{125}\text{I-FCS}}$$

C. The Standard Curve of Apparent Molecular Weight versus Relative Mobility:

Protein markers of known molecular weights were labelled with carrier-free ^{125}I, at a concentration of 0.1 mg of protein per 0.3 mCi of ^{125}I, by the chloramine T procedure. After dialysis, the radiolabelled proteins were diluted to a concentration of 1 µg per ml with PBS. About 0.1 µg of each protein marker was analyzed by SDS-PAGE on the 10% gel system as mentioned in Chapter II, Section III:L,2. The distance travelled by the tracking dye of each sample was carefully measured with a ruler and was recorded in millimetres. After the cpm of each fraction of the individual gel was determined by the gamma counter, the fraction where the protein peak had a maximum was recorded and was converted into distance by multiplying the fraction number by 2 mm. The relative mobility of each protein marker was calculated as follows:

$$\text{relative mobility} = \frac{\text{distance of peak travelled}}{\text{distance of dye travelled}}$$

Then, the known molecular weight of each protein was plotted on the logarithmic scale versus its individual relative mobility.

D. Radioiodination of Target Cells in the Presence of IgE:

Target cells at an appropriate concentration (5×10^6 for RMC or 2×10^7 for RBL cells in 1 ml of THM/BSA) were incubated with 10 µg of IgE at 37°C for two hours. The cells were centrifuged at 200 x g for 5 minutes. The supernatant was discarded and the cell pellet was

resuspended in THM/BSA. The washing procedure was repeated twice by centrifugation. The cells were then ready for surface labelling as described in Chapter II, Section III:J.

E. To Remove The Receptors for IgE from the Detergent Extract of Target Cells:

Target cells were surface labelled and solubilized with the detergent as described before. The receptors were removed from the cell extract by either one of the following methods.

1. By pre-precipitation

Each aliquot of the cell extract, equivalent to 2.5×10^6 RMC or 1×10^7 RBL cells, was incubated with 10 μg of IgE at 37°C for 30 minutes. About 0.4 mg of rabbit anti-rat IgE was added to the sample and the incubation was carried on for another 30 minutes. Then, 0.1 ml of Protein A-Sepharose in PBS/0.1% NP-40 was used to insolubilize the antigen-antibody complexes of each sample by incubating the mixture at room temperature over one and a half hours with constant mixing. The supernatant was removed after centrifugation and was transferred to a clean tube for further experiments.

2. By affinitive adsorption

The receptors were removed from the cell extract by adsorbing the latter with IgE-Sepharose. The IgE-Sepharose preparation was routinely prepared in Dr. A. Froese's laboratory. About 100 - 200 mg of a semi-purified (Bio-Gel P-300 chromatography) preparation of IgE were coupled with 10 ml of packed cyanogen bromide-activated Sepharose-CL-4B. The procedures were similar to the ones described in Chapter II, Section III:D. The IgE-Sepharose preparation was kept at 4°C in PBS containing 0.1% sodium azide and was equilibrated with

PBS/0.1% NP-40 before use. Each aliquot of the cell extract was incubated with IgE-Sepharose at a volume ratio of 1 : 1 at 4°C over one hour. The mixture was then centrifuged at 1000 rpm for 8 minutes. The supernatant was transferred to a clean tube for further experimenting.

F. Isolation of Receptors for IgE by means of Affinity Chromatography:

The receptors for IgE were isolated from the target cell extract either in their free form or as IgE-receptor complexes. The former will be referred to as the KSCN eluate and the latter as the DNP-ONa eluate.

1. Preparation of the KSCN eluate

The isolation of free receptors for IgE was performed according to the method described by Conrad and Froese (1978a). The cell extract was incubated with IgE-Sepharose in PBS/0.1% NP-40 at a volume ratio of 1 : 1 at 4°C for one hour. After centrifugation at 1000 rpm for 8 minutes, the supernatant was removed and the Sepharose was mixed in 3 ml of PBS/0.1% NP-40 and the mixture was packed into a mini-column made from a pasteur pipette. The column was washed with 15 ml of PBS/0.1% NP-40 or until the effluent contained less than 2,000 cpm. Then the bound receptors were eluted from the column with 3 - 5 ml of KSCN in PBS/0.1% NP-40. The effluent was collected at about 20 drops per fraction. After the entire volume of the KSCN solution had passed through the column, it was filled with PBS/0.1% NP-40 again. The fractions collected were counted for their radioactive contents. The fractions with the highest cpm were pooled and the KSCN eluate was then dialyzed against PBS/0.1% NP-40 at 4°C overnight prior to being used.

2. Preparation of the DNP-ONa eluate

The IgE-receptor complexes were isolated according to the method described by Conrad and Froese (1978a). The preparation of $DNP_{7.7}$-IgE was obtained from Dr. A. Froese's laboratory and was labelled with carrier-free ^{131}I by the chloramine T procedure. The cells were first surface labelled with carrier-free ^{125}I. After washing with 10 ml of cold THM/BSA buffer, the cells were resuspended in 1 ml of buffer and were incubated with 5.7 µg of ^{131}I-labelled $DNP_{7.7}$-IgE at 37°C for an hour. Additional cold $DNP_{7.7}$-IgE was added to the mixture (300 µg per sample) and the incubation was continued for another hour. Then, the cell suspension was carefully layered on top of 2 ml of FCS and the tube was centrifuged at 1,000 rpm for one minute. After all of the supernatant had been aspirated, the cell pellet was resuspended and washed in THM/BSA by centrifugation at least twice. The cells were then solubilized as described in Chapter II, Section III:J. The cell extract was incubated with 0.25 - 0.30 ml of anti-DNP coupled to Sepharose at 4°C for one hour. The anti-DNP-Sepharose preparation was also obtained from Dr. A. Froese's laboratory. At the end of the incubation, the mixture was centrifuged at 1,000 rpm for eight minutes and the supernatant was discarded. About 3 ml of PBS/0.1% NP-40 were mixed with the gel and the mixture was packed into a mini-column as described before. The column was washed with 15 ml of PBS/0.1% NP-40. Then, 3 ml of 0.1 M 2,4-dinitrophenolate in 0.1 M phosphate buffer, containing 0.1% NP-40 (DNP-ONa) was used to elute the IgE-receptor complexes from the column. The effluent was collected at 10 drops per fraction. The fractions were counted for their radioactive contents (both ^{125}I and ^{131}I) by a Beckman Gamma Counter (Model 300, Beckman

Instrument Co., Fullerton, Calif.). Those with the highest cpm (^{125}I and ^{131}I) were pooled. The DNP-ONa eluate was then dialysed against the appropriate buffer prior to being used.

G. KSCN Eluates Binding to Different Sepharose Conjugates:

After dialysis against PBS/0.1% NP-40, the KSCN eluates were incubated with either IgE-Sepharose or anti-RBL coupled Sepharose at $4^{\circ}C$ for one hour at a volume ratio of 1 : 1. The IgE-Sepharose preparation was obtained as mentioned before whereas anti-RBL-Sepharose was prepared according to the procedures described in Chapter II, Section III:D. About 14 - 15 mg of the immunoglobulin fraction of anti-RBL were reacted with each ml of packed, activated Sepharose-CL-4B. The coupling efficiency was about 75 - 80%. The affinity gels were stored at $4^{\circ}C$ in PBS, containing 0.1% sodium azide, and were equilibrated with PBS/0.1% NP-40 before use. At the end of the incubation, the gels were centrifuged at 1,000 rpm for eight minutes. After the supernatant had been removed, the gels were washed at least five times with PBS/0.1% NP-40 by centrifugation. Then, bound surface antigens were eluted from the gels by heating the latter at $100^{\circ}C$ for 90 seconds in the presence of 0.2 ml of sample buffer containing 2% SDS. The eluted samples were then analyzed by SDS-PAGE on 10% gels.

H. Absorption of Sera with RMC or RBL Cells:

About 1 ml of anti-RMC (1 : 10) was absorbed with 1 ml of packed RBL cells ($1 - 2 \times 10^8$), pre-washed with PBS. The absorption procedure was carried out at $4^{\circ}C$ for one hour with constant rotation of the tube. After centrifugation at 200 x g for ten minutes, the cell pellet was discarded and the supernatant was absorbed with a new batch of RBL cells. The anti-RMC serum was absorbed with RBL cells three times and was

designated as anti-RMC_{bas}. The anti-RBL cell serum was absorbed with purified RMC the same way as above except that the amount of RMC used was 1×10^7 per absorption. Since about 1×10^7 RMC could be obtained from 4 - 5 rats, it was technically difficult to obtain $1 - 2 \times 10^8$ purified RMC. Therefore, less RMC were used for the absorption which was repeated for another four times and the resultant serum was designated as anti-RBL_{mas}. After the final absorption, both anti-RMC_{bas} and anti-RBL_{mas} were clarified by centrifugation at 50,000 x g for one hour at 4°C. The immunoglobulin fractions of both sera were isolated by precipitation with saturated $(NH_4)_2SO_4$ as described in Chapter II, Section III:B. The optical density of each of the two immunoglobulin preparations was measured and its protein concentration was determined by using an extinction coefficient of 13.6 ($E^{1\%}_{280\ nm}$).

IV. RESULTS AND DISCUSSIONS

A. Surface Antigens of RMC and RBL Cells:

Initial experiments were aimed at comparing the total cell surface proteins and glycoproteins present on RMC and RBL cells with those precipitated by the appropriate antisera. For this purpose, RMC or RBL cells were first surface radioiodinated and then solubilized with 0.1% or 0.5% NP-40 in PBS. A small portion of the detergent-extract was added to 0.2 ml of 0.0625 M Tris buffer containing 2% SDS (pH 6.8) and the mixture was heated at 100°C for 90 seconds. A second aliquot of the same extract was precipitated with the appropriate antiserum. Protein A-Sepharose was used to insolubilize the immune complexes which were subsequently eluted from the absorbent with the

sample buffer containing 2% SDS at 100°C. The whole cell extract and the antiserum-bound surface antigens were then analyzed with SDS-PAGE on 10% gels. In order to find out if reduction would affect the results of the analysis, duplicate samples of the above were prepared and were reduced by adding 10 µl of 2-mercaptoethanol to each sample in the sample buffer at 100°C prior to the analysis. The results were compared with those obtained from the non-reduced samples. It was found that reduction of the samples did not alter any of the profiles of the surface antigens. Hence, all subsequent analyses were carried out under non-reducing conditions. The non-reduced sample showed that the ^{125}I-labelled membrane proteins of RMC covered the area between fractions 3 and 45. It is interesting to note that the majority of surface proteins or glycoproteins migrated with mobilities corresponding to apparent molecular weights ranging from 30,000 to 75,000 daltons (Fig. 11a). The anti-RMC_{abs} serum appeared to react with many of these components. In addition, the antiserum precipitated two surface antigens with one peak in fraction 3 (peak 1) and the other in fraction 12 (peak IV), both of which were prominent enough to show up as two distinct peaks in the whole cell extract. All these peaks were specifically associated with anti-RMC_{abs} since the normal rabbit serum did not precipitate any of the RMC surface antigens under the same conditions. The specificity of anti-RMC_{abs} was further demonstrated indirectly when rabbit anti-rat sarcoma cell (anti-S) sera were used to precipitate the ^{125}I-labelled RMC detergent-extract. Both the unabsorbed and absorbed anti-S sera reacted only to a limited extent with the RMC surface extract (Fig. 11b), which was much less than that involving anti-RMC_{abs}. To further check the specificity of the

Fig. 11 10% SDS-PAGE Analysis of RMC Surface Antigens

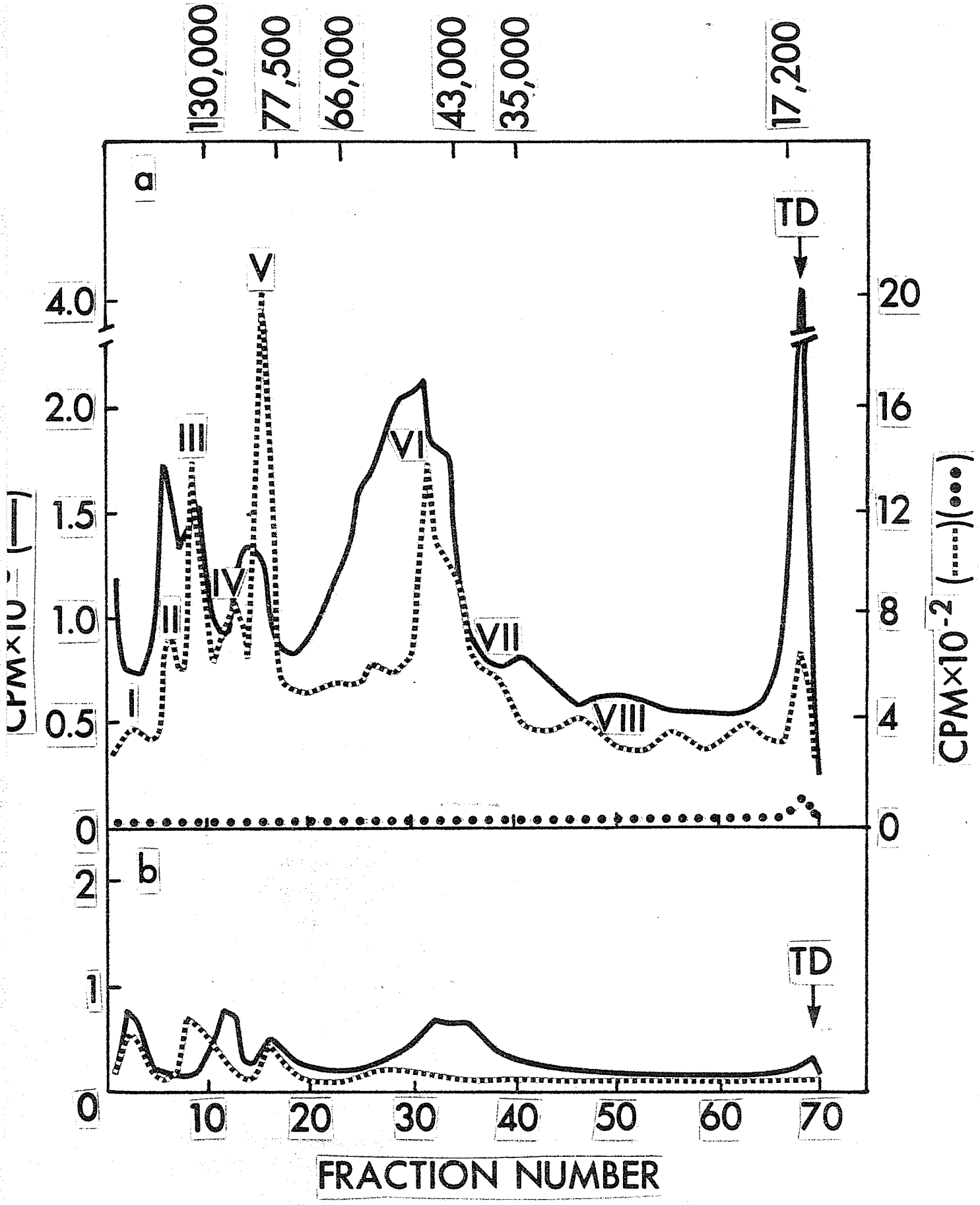
Two batches of RMC (5×10^6 cells per batch) were labelled with carrier-free ^{125}I and solubilized with PBS/0.1% NP-40. The pooled cell extract was dialyzed versus PBS/0.1% NP-40 before being divided into five aliquots.

(a) A small portion of one of the aliquots was added to 0.2 ml of 0.0625 M Tris buffer containing 2% SDS (pH 6.8) (————). A second aliquot was incubated with 0.4 mg of anti-RMC_{abs} (- - - -) and the third with NRS (· · ·) of an equal amount at 37°C for 30 mins.

(b) The fourth aliquot was incubated with anti-S (———) and the fifth with anti-S_{abs} (- - -) the same way as in (a).

Protein A-Sepharose was used to insolubilize the immune complexes which were subsequently eluted from the absorbent with the sample buffer containing 2% SDS at 100°C. All samples were analyzed with SDS-PAGE.

Protein markers: β-galactosidase (130,000), lactoperoxidase (77,500), human albumin (66,000), ovalbumin (43,000), pepsin (35,000) and myoglobin (17,000)


130,000
77,500
66,000
43,000
35,000
17,200
a
TD
V
4.0
20
2.0
16
III
VI
1.5
12
IV
II
1.0
8
VII
I
0.5
4
VIII
0
0
b
2
TD
1
0
10
20
30
40
50
60
70
FRACTION NUMBER
CPM×10⁻² (••••)(••)

anti-RMC serum the following experiments were carried out. Rat lymph node (LN) cells were radioiodinated and solubilized the same way as RMC. The LN cell extract was divided into several aliquots, two of which were reacted with anti-RMC and anti-RMC_{abs} separately. The dissolved immune complexes were analyzed with SDS-PAGE. In addition, a portion of the LN cell surface extract was also analyzed. The results showed that the LN cell surface components spread throughout almost the entire length of the gel (Fig. 12a) and would thus represent molecules of molecular weight (m.w.) ranging between 20,000 to 200,000 daltons. The anti-RMC serum precipitated predominantly components found between fractions 20 and 25 (Fig. 12b). No major surface antigens of RMC had a corresponding m.w., suggesting that a molecule of corresponding antigenicity was present in RMC but that its frequency of occurrence was much lower than that on LN cells. Absorption removed most of the antibodies from anti-RMC_{abs} which still precipitated a few LN cell surface antigens of higher molecular weight found between fractions 3 and 15 (Fig. 12c). Based on the data obtained from the cytotoxicity studies (Fig. 6), one has to conclude either that anti-RMC_{abs} was no longer cytotoxic to LN cells or that the antibodies were too few in concentration to yield detectable level of cytotoxicity. However, it is uncertain if the LN cell surface antigens found between fractions 3 and 15 were similar or identical to RMC surface antigens found in the same area (Fig. 11a: peaks I, II, III & IV) even though they reacted to the same antiserum. In view of the fact that the anti-S_{abs} serum also precipitated RMC surface antigens that migrated to the same regions (Fig. 11b), they could represent either surface antigens which generally reacted with natural rab-

Fig. 12 10% SDS-PAGE Analysis of Lymph Node Cell Surface Antigens Precipitated by Anti-RMC Sera

Two batches of lymph node cells (2×10^7 cells per batch) were radioiodinated and solubilized the same way as described in the legend of Fig. 11. The pooled extracts were divided into four aliquots.

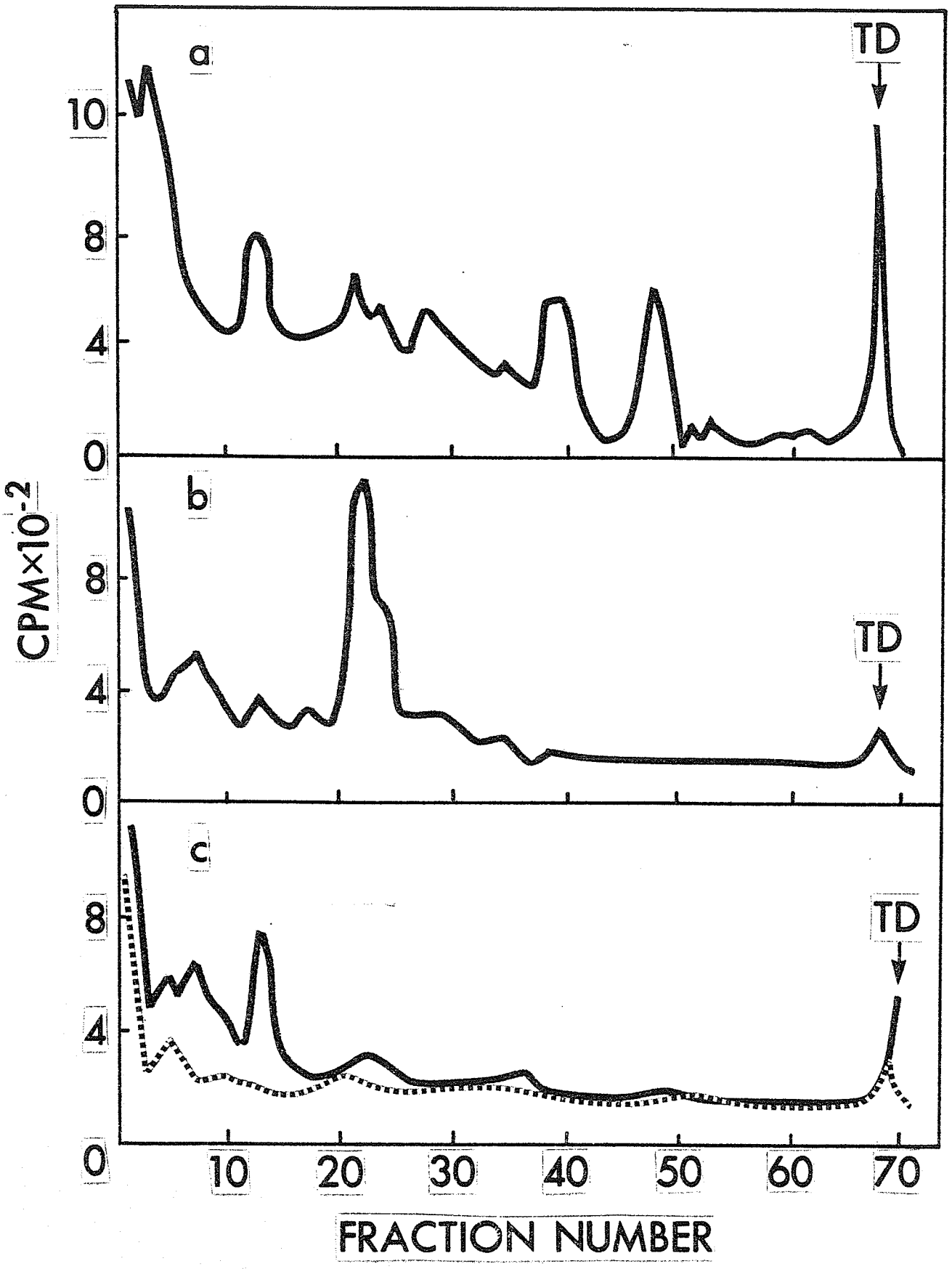
(a) A small portion of the first aliquot was dissolved in 0.2 ml of the sample buffer containing 2% SDS.

(b) The second aliquot was incubated with 0.4 mg of anti-RMC.

(c) The third aliquot was incubated with 0.4 mg of anti-RMC_{abs} (————).

The fourth aliquot was first incubated with 10 µg of IgE and subsequently with 0.4 mg of rabbit anti-rat IgE (- - - - -).

All incubations were carried out at 37°C for a period of 30 mins except for (c) where the mixture was incubated with anti-IgE for another 30 mins. All immune complexes were treated and analyzed as described in Fig. 11 by SDS-PAGE.


a
b
c
TD
TD
TD
10
8
4
0
8
4
0
8
4
0
CPM×10-2
10
20
30
40
50
60
70
FRACTION NUMBER

bit antibodies or common determinants that were present on the surface of rat lymphoid cells including RMC but were absent from liver cells. Since normal rabbit serum did not precipitate these RMC surface components (Fig. 11a), the first possibility can probably be eliminated. All the other RMC surface components that were associated with anti-RMC $_{abs}$ were indeed confined to the surface of RMC only, since no LN cell surface components of similar electrophorectic mobilities were precipitated by the same antiserum.

When the total radio-labelled cell surface proteins or glycoproteins and those reacting with anti-RBL$_{abs}$ were analyzed by SDS-PAGE, the results shown in Fig. 13 were obtained. The anti-RBL$_{abs}$ serum reacted with most of the RBL cell surface components (Fig. 13a) spanning a mobility range between fractions 5 and 50. At least seven distinct surface antigens were precipitated by the antiserum. By comparison, neither the anti-S serum nor the normal rabbit serum (NRS) precipitated any of the RBL cell surface components (Fig. 13b). The specificities of both anti-RBL and anti-RBL $_{abs}$ were also assessed by analyzing the LN cell surface antigens capable of reacting with these antisera. The anti-RBL serum precipitated a substantial number of LN cell surface components which appeared between fractions 5 and 40 (Fig. 14a). When the result was compared with that in Fig. 12b, it was obvious that there were far more cross-reacting antibodies present in anti-RBL than there were in anti-RMC. Absorption with liver cells did remove most of these antibodies from anti-RBL. However, the absorbed serum still precipitated several LN cell surface antigens that migrated to the region between fractions 3 and 30 (Fig. 14b). The corresponding antibodies were probably not involved in cell lysis since anti-RBL$_{abs}$

Fig. 13 10% SDS-PAGE Analysis of RBL Cell Surface Antigens

Two batches of RBL cells (2×10^7 cells per batch) were radioiodinated and solubilized. The detergent-extracts were pooled and redivided into four aliquots.

(a) The first aliquot was dissolved the same way as described in the legend of Fig. 11 (————).

The second aliquot was incubated with 0.4 mg of anti-RBL_{abs} (- - - -).

(b) The third aliquot was incubated with 0.4 mg of anti-S (————).

The fourth aliquot was incubated with NRS of the same amount as above (- - - -).

All incubations were carried out at 37°C for 30 mins. The bound materials were eluted from Protein A-Sepharose and analyzed by SDS-PAGE.

Protein marker : see legend, Fig. 11.

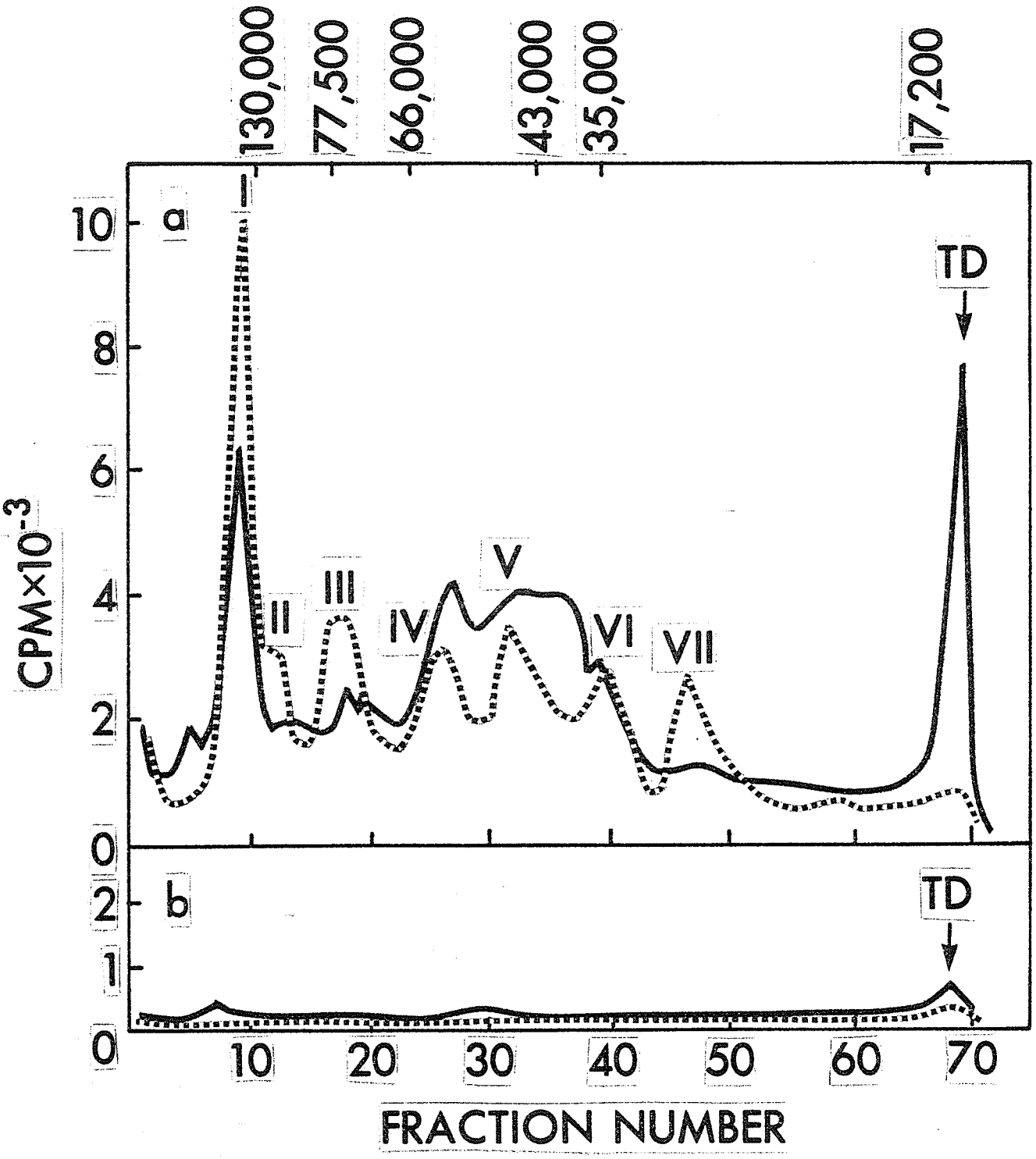

130,000
77,500
66,000
43,000
35,000
17,200
a
b
TD
TD
II
III
IV
V
VI
VII
CPM×10-3
10
8
6
4
2
0
2
1
0
10
20
30
40
50
60
70
FRACTION NUMBER

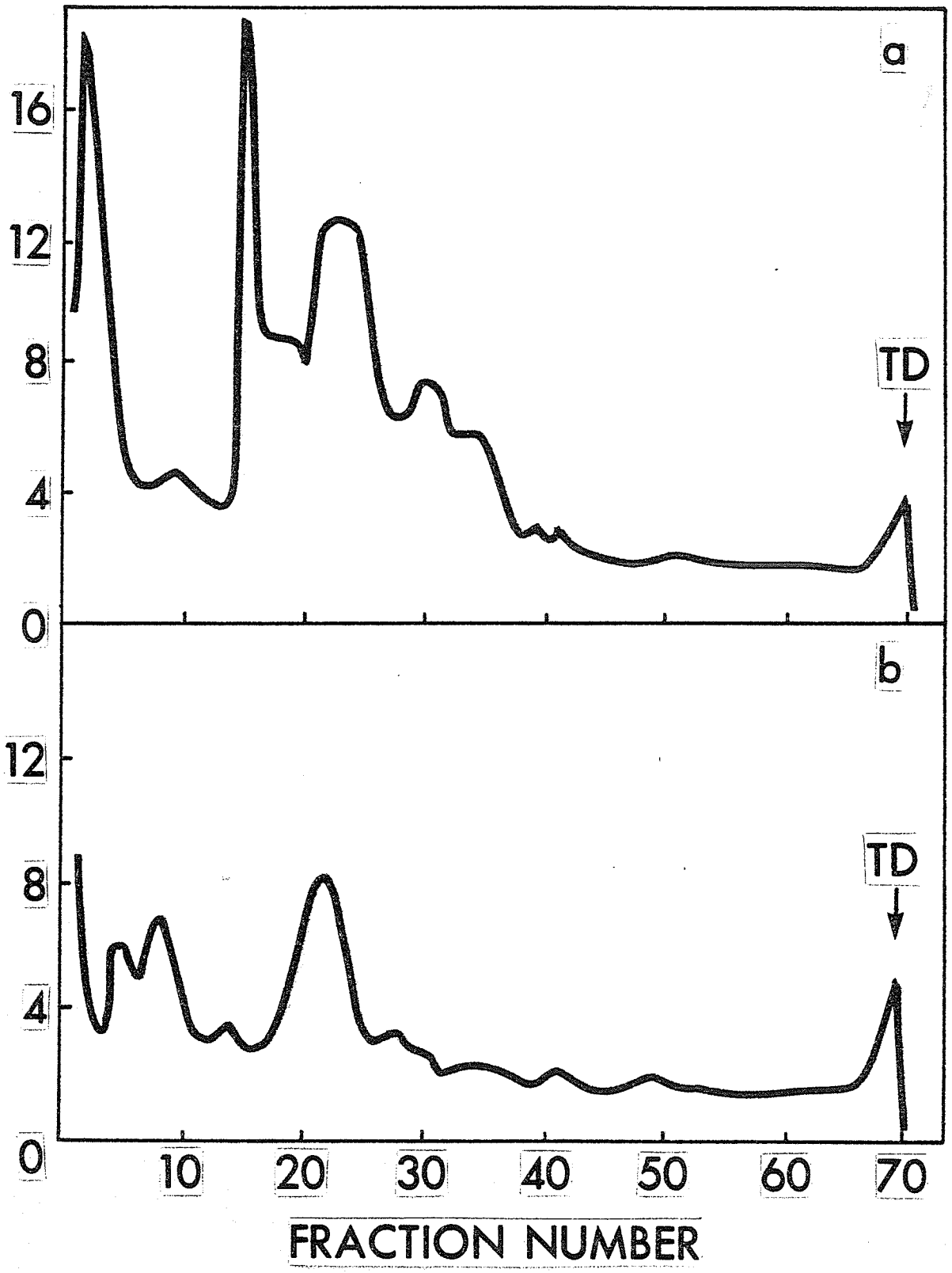
Fig. 14 10% SDS-PAGE Analysis of Lymph Node Cell Surface Antigens Precipitated by the Anti-RBL Sera

Lymph node cells (2×10^7) were radioiodinated and solubilized under the same conditions described in the legend of Fig. 12. The extract was divided into two aliquots.

(a) One aliquot was incubated with 0.4 mg of anti-RBL.

(b) The other aliquot was incubated with anti-RBL_{abs} of the same amount as above.

All incubations were carried out at 37°C for 30 mins. The antigen-antibody complexes were insolubilized with Protein A-Sepharose and were eluted from the adsorbent for subsequent analysis by SDS-PAGE.


a
16
12
8
4
0
TD
b
12
8
4
0
TD
10
20
30
40
50
60
70
FRACTION NUMBER

was no longer cytotoxic to LN cells (Fig. 6). The peak appearing in fraction 22 (Fig. 14b) could represent certain LN cell surface antigens that cross-reacted with anti-RBL_{abs}, but was not present in any significant amount on RBL cells (Fig. 13a). Components found between fractions 3 and 15 (Figs. 13a & 14b) are most likely surface antigens that are shared by LN cells, RBL cells and RMC but not by liver cells. Apart from those mentioned above, peaks appearing between fractions 15 and 50 of both Figs. 11a and 13a probably represented certain specific antigens present on the surface of RMC and/or RBL cells.

It has been reported that fetal calf serum (FCS) could bind to the surface of cultured cells and thereby affect the properties of these cells (Kerbel and Blakeslee, 1976; Opitz et al., 1977). Since RBL cells were cultured in a medium containing 15% FCS which was required for supporting the cell growth, it was possible that FCS might still adhere to the cell surface even after thorough washing procedures. Hence, its presence during the time of immunization might induce anti-FCS antibodies or its adherence to the RBL cell surface during radioiodination might alter the results of the analysis. These possibilities were investigated by three different studies. Fetal calf serum was first radioiodinated by the chloramine T procedure. Aliquots of ^{125}I-FCS were allowed to react with the immunoglobulin preparation of rabbit anti-bovine serum albumin (anti-BSA), NRS or anti-RBL_{abs}. The antigen-antibody complexes were insolubilized with Protein A-Sepharose. After thorough washing, the radioactivity bound by Protein A-Sepharose was counted. The results were calculated and compared. In order to assure that any binding of ^{125}I-FCS was not due to Protein A-Sepharose, a control with only ^{125}I-FCS and the latter was used. While Protein

A-Sepharose alone bound very little ^{125}I-FCS, all the rabbit sera including NRS were reactive to the antigen to different extents (Table VIa). The results indicated that anti-RBL$_{abs}$ might contain some antibodies to FCS even though natural anti-bovine antibodies might be generally present in rabbit sera. If this was so, then it would mean that FCS might bind to the surface of RBL cells during tissue culturing and might subsequently be radioiodinated. As a consequence, some of the peaks observed in Fig. 13 might represent components belonging to FCS. In order to clarify these points, the following experiments were performed. First of all, a fixed amount of ^{125}I-FCS was incubated with RBL cells of known concentrations at 4°C, 25°C and 37°C for one hour. After washing through 2 ml of FCS followed by two subsequent washings in PBS/BSA, the cell pellets were counted for their radioactive content. The results clearly demonstrated that ^{125}I-FCS did not significantly bind to RBL cells (Table VIb). Next, ^{131}I-labelled RBL cell surface antigens bound by anti-RBL$_{abs}$ were analyzed on the same gel with ^{125}I-FCS precipitated by different rabbit sera. While ^{125}I-FCS alone gave one single peak in fraction 28 (Fig. 15a), its precipitates by anti-RBL$_{abs}$ (Fig. 15b) and NRS (Fig. 15c) also migrated to the same region on 10% gels. The results showed that the peak in fraction 28 did not coincide with any of the RBL cell surface antigens precipitated by anti-RBL$_{abs}$. The third study was to investigate the effect of FCS in excess on the reaction between anti-RBL$_{abs}$ and its antigens. This was done by deliberately adding FCS equal to a final concentration of 20% to the RBL cell extract before the addition of anti-RBL$_{abs}$. The bound surface antigens were eluted from Protein A-Sepharose and were analyzed on the same gel with ^{131}I-labelled RBL cell surface antigens,

TABLE VIa

BINDING OF ^{125}I-FCS BY VARIOUS ANTISERA*

Antiserum	µg of ^{125}I-FCS Bound
Anti-BSA	0.300
Anti-RBL$_{abs}$	0.076
NRS	0.020
Protein A-Sepharose	0.002

* Aliquots of ^{125}I-FCS were incubated with the immunoglobulin preparations of rabbit anti-bovine serum (anti-BSA), NRS and anti-RBL$_{abs}$, all of the same protein concentration (0.4 mg of IgG/1 µg of ^{125}I-FCS), separately. The immune complexes were insolubilized with an equal amount of Protein A-Sepharose (0.1 ml per tube). After thorough washing, the samples were counted for their radioactive contents.

TABLE VIb

BINDING BETWEEN ^{125}I-FCS AND RBL CELLS**

Temperature	% of ^{125}I-FCS Bound
4°C	0.25
25°C	0.30
37°C	0.56

** RBL cells (3×10^7) were washed twice with THM. The cell pellet was resuspended with the same buffer and was divided into 3 aliquots. Each aliquot was incubated with 1 µg of ^{125}I-FCS at different temperatures for 1 hour. After thorough washing, cells were counted for their radioactive contents.

Fig. 15 10% SDS-PAGE Analysis of ^{125}I-FCS

RBL cells (3×10^7) were labelled with ^{131}I and were solubilized in PBS/0.1% NP-40. The cell extract, in 1 ml volume, was allowed to react with 1.2 mg of anti-RBL_{abs} at 37°C for 30 mins. The antigen-antibody complexes were insolubilized with 0.3 ml of Protein A-Sepharose. After the bound materials were eluted, they were divided into 3 aliquots.

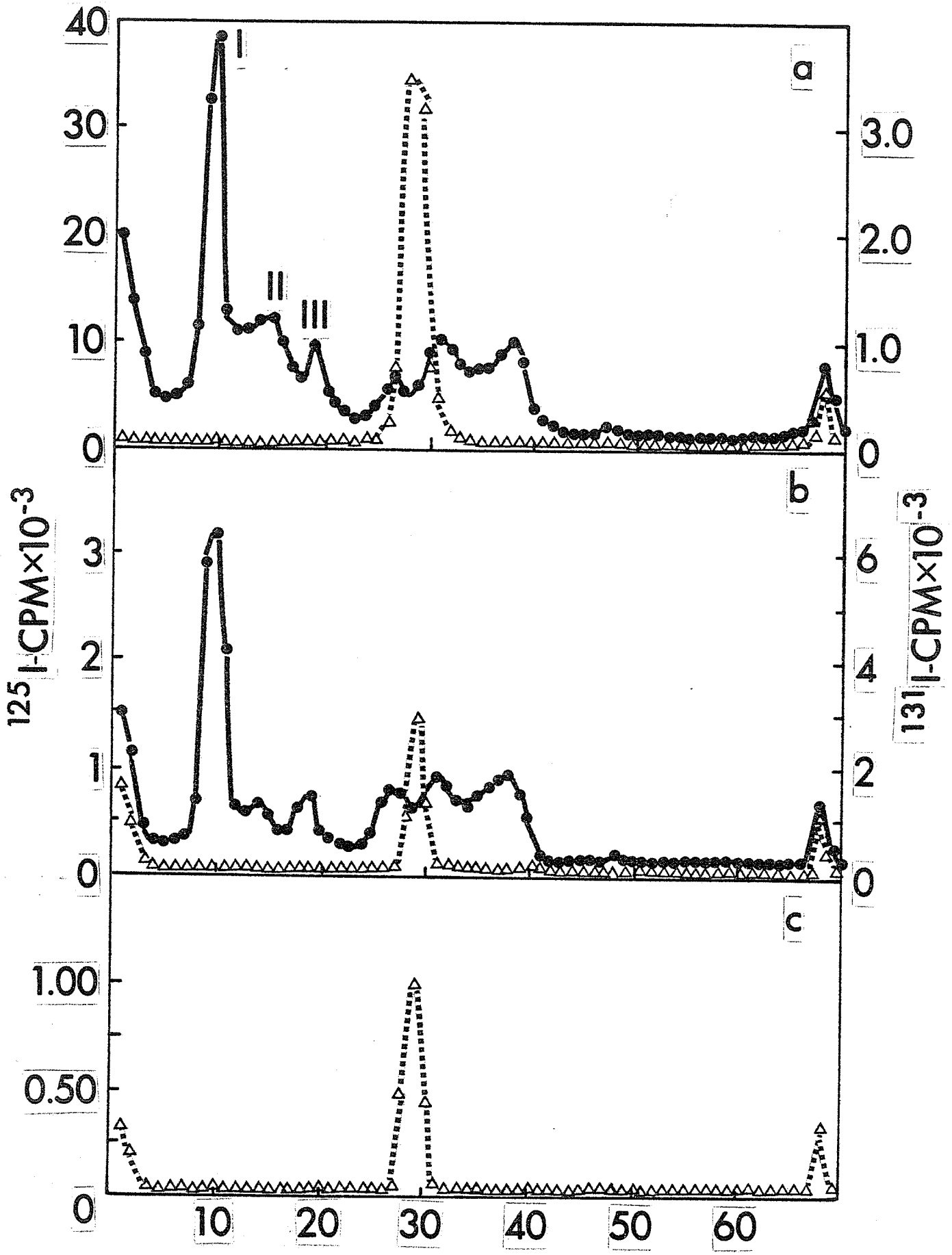
(a) One of the above aliquots was analyzed on the same gel with 0.2 µg of ^{125}I-FCS.

(b) Another aliquot was analyzed on the same gel with 0.01 µg of ^{125}I-FCS, previously precipitated by anti-RBL_{abs} (0.4 mg).

(c) About 0.005 µg of ^{125}I-FCS, previously precipitated by NRS (0.4 mg) were analyzed by SDS-PAGE.

RBL cell surface antigens : (●————●)

^{125}I-FCS : (Δ- - - - -Δ)


a
b
c
I
II
III
40
30
20
10
0
3.0
2.0
1.0
3
2
1
6
4
2
1.00
0.50
10
20
30
40
50
60
125I-CPM×10-3
131I-CPM×10-3
FRACTION NUMBER

bound by anti-RBL_{abs} in the absence of FCS. The two patterns obtained were very similar, indicating that FCS did not interfere with the binding between the antiserum and its antigens (Fig. 16). However, peaks I, II and III of both patterns were different from the corresponding peaks observed in Figs. 13a and 15a. Peak I, which was usually seen as the most dominant peak, had been diminished, whereas peak II, which was usually a minor component, was drastically increased in intensity. Peak III remained at about the same intensity, but appeared to have migrated slightly faster than the corresponding peak observed in Figs. 13a and 15a. The reason for these changes is not clear. It is conceivable that they may be due to artifacts brought about by overloading the gel with proteins. Such an artifact has been observed by others (Conrad and Froese, 1978a).

It had become certain that anti-RMC_{abs} precipitated at least eight surface components of RMC while anti-RBL_{abs} reacted with at least seven components of the RBL cell surface. Apart from those that appeared between fractions 3 and 15, all the rest of the protein peaks shown in Figs. 11a and 13a represented specific surface antigens of RMC and RBL cells, respectively. In order to determine the apparent m.w. of these peaks, protein markers of known m.w. were ^{125}I-labelled and analyzed on individual gels with 10% SDS-PAGE. Their electrophorectic mobilities were calculated and were plotted against their corresponding log m.w. A straight line was obtained which could be used for determining the apparent m.w. of proteins within the range from 35,000 to 130,000 daltons (Fig. 17) under the same analytical conditions. Based on this, the apparent m.w. of the peaks which appear in Figs. 11a and 13a were determined by reading the individual mobility against the corresponding

Fig. 16 10% SDS-PAGE Analysis of RBL Cell Surface Antigens Reacting with Anti-RBL_{abs} in the Presence or Absence of FCS

The extract of ^{131}I-labelled RBL cells (1×10^7) reacting with anti-RBL_{abs} (●——●) (see the legend of Fig. 15) was analyzed on the same gel with the surface components of ^{125}I-labelled RBL cells (1×10^7) reacting with anti-RBL_{abs} (0.4 mg of IgG) in the presence of 20% FCS (Δ- - - -Δ). Each radiolabel contained about 100,000 cpm. This was done by transferring an appropriate volume of each of the ^{125}I or ^{131}I-labelled samples which gave the specific count of the above to another tube. The samples of the radiolabels were then mixed together and analyzed on the same gel.

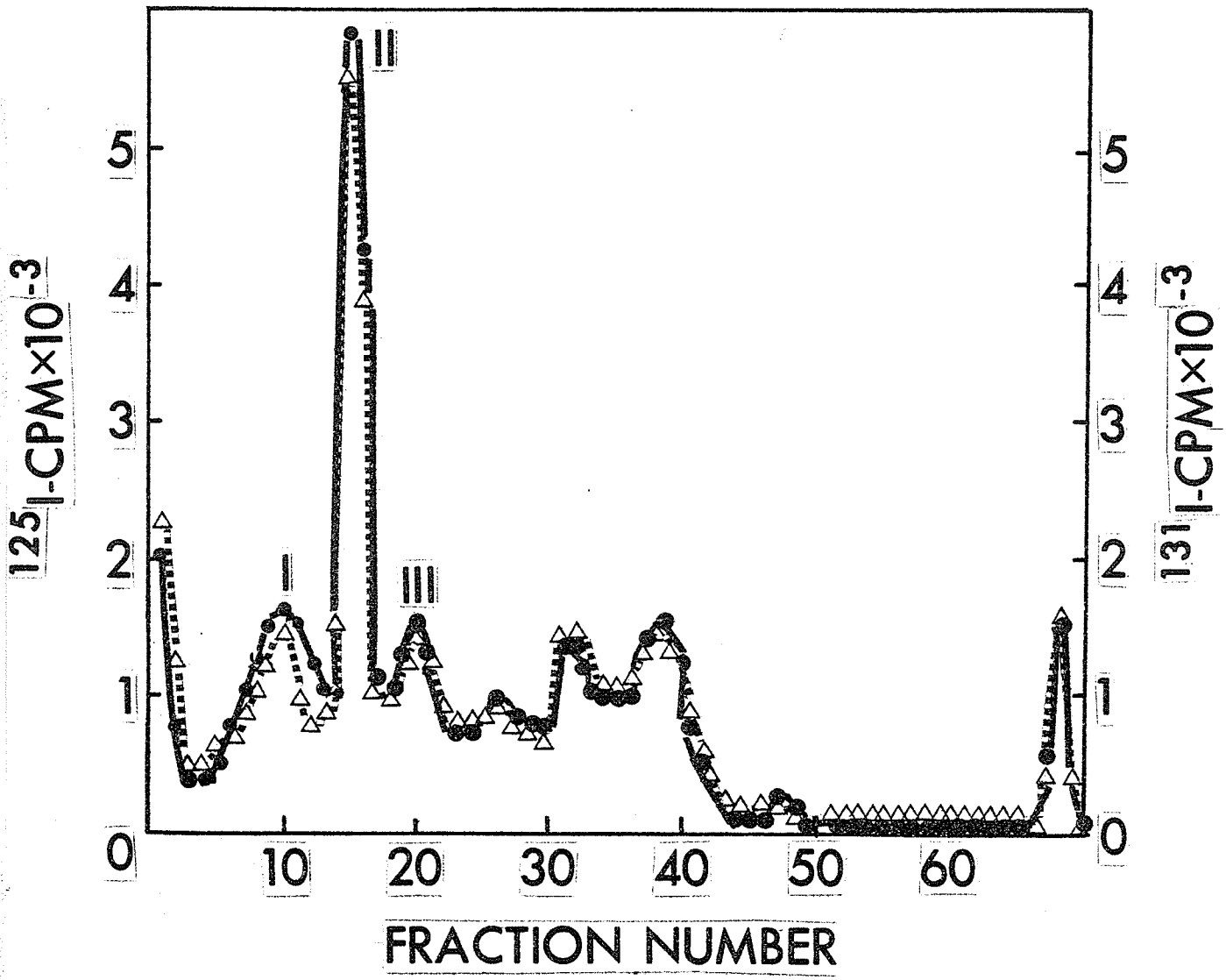
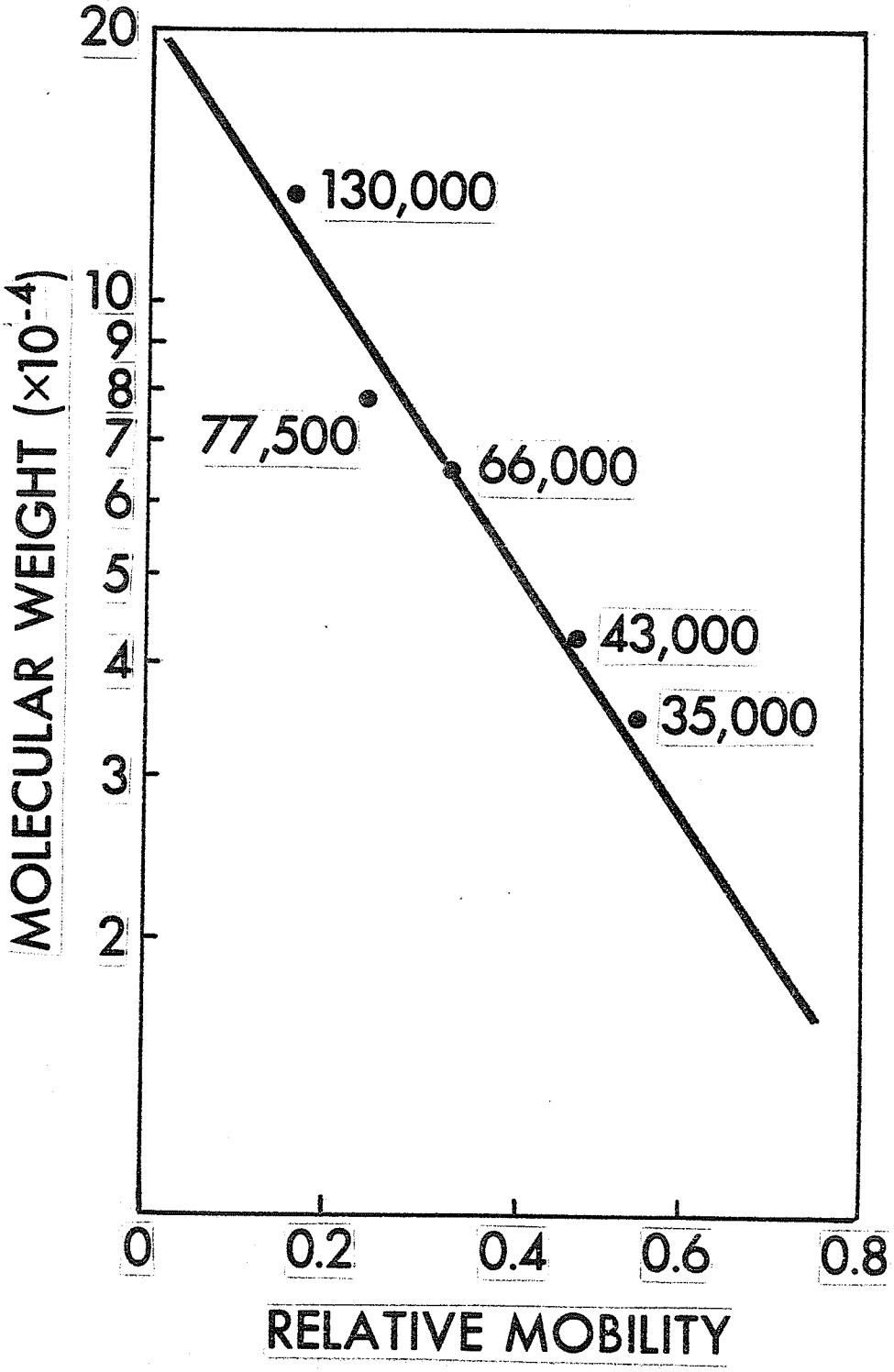

125I-CPM×10-3
131I-CPM×10-3
I
II
III
0
1
2
3
4
5
10
20
30
40
50
60
FRACTION NUMBER

Fig. 17 The Standard Curve of Apparent Molecular Weight versus Relative Mobility

Protein markers: β-galactosidase (130,000)
lactoperoxidase (77,500)
human albumin (66,000)
ovalbumin (43,000)
pepsin (35,000)

All of the above proteins were labelled with ^{125}I and were analyzed by 10% SDS-PAGE on separate gels but under the same conditions. The fraction number where the maximum of the peak appeared was recorded and converted into relative mobility as described in METHODS. The molecular weight of each protein was plotted versus its relative mobility on a semi-logarithmic scale.


20
10
9
8
7
6
5
4
3
2
MOLECULAR WEIGHT (×10⁻⁴)
130,000
77,500
66,000
43,000
35,000
0
0.2
0.4
0.6
0.8
RELATIVE MOBILITY

m.w. on the standard curve. The results were recorded and shown in Table VIIa for RMC surface antigens and Table VIIb for RBL cell surface components. By comparing the data presented in both tables, it was found that there were several RMC surface components (associated with anti-RMC_{abs}) having almost identical electrophorectic mobilities as some of the RBL cell membrane antigens (bound by anti-RBL_{abs}). Most noticeably, peaks III and VI of RMC origin had the same mobilities as peaks I and V from the RBL cell surface. In view of the fact that both anti-RMC_{abs} and anti-RBL_{abs} had been shown to cross-react with these two cell types previously, the similarities in electrophorectic behavior of their antigens could possibly suggest that the antisera might react with identical or similar molecules, present on the surface of both cells. Hence, a series of studies was carried out in order to search for these common antigens. The results are presented in the following section.

B. Characterization of the Common Surface Antigens of RMC and RBL Cells:

The first set of experiments was designed to establish evidence that both RMC and RBL cells were associated with certain surface antigens that have similar electrophorectic mobilities. The surface antigens of RMC or RBL cells were surface-labelled with either ^{125}I or ^{131}I and the detergent-solubilized extracts were allowed to react with appropriate antisera. The antigen-antibody complexes were isolated either by precipitation with a goat anti-rabbit immunoglobulin serum or by insolubilization with Protein A-Sepharose. After thorough washing and dissolving the complexes in the sample buffers containing 2% SDS, the ^{125}I-labelled complexes were analyzed on the same gel with ^{131}I-labelled

TABLE VIIa

RMC SURFACE ANTIGENS PRECIPITATED BY ANTI-RMC$_{abs}$

Protein Peak	Fraction No.	Apparent Molecular Weight
I	3	175,000
II	5	160,000
III	9	130,000
IV	13	105,000
V	16	85,000
VI	32	45,000
VII	39	35,000
VIII	46	<35,000

TABLE VIIb

RBL CELL SURFACE ANTIGENS PRECIPITATED BY ANTI-RBL$_{abs}$

Protein Peak	Fraction No.	Apparent Molecular Weight
I	9	130,000
II	12	110,000
III	18	78,000
IV	26	55,000
V	32	45,000
VI	40	34,000
VII	47	<34,000

complexes. In one of these studies, the complexes consisted of anti-RBL_{abs} reacted with ^{125}I-labelled RBL cell extract and ^{131}I-labelled RMC extract associated with anti-RMC_{abs}. Results from the 5% SDS-PAGE analysis revealed that two of the five precipitated RMC surface components migrated in fractions also occupied by RBL cell surface antigens (Fig. 18). The similaries appeared in fractions 20 and 23. The receptor for IgE (R) on the RMC surface had been previously characterized and was shown to migrate to fraction 23 under the same experimental conditions (Conrad and Froese, 1976). The ^{131}I-labelled RBL cell surface component precipitated by IgE-goat anti-rat IgE had a similar mobility as one of the ^{125}I-labelled RBL cell surface antigens associated with the cross-reacting anti-RMC_{abs} (Fig. 19a). Both components of ^{125}I and ^{131}I-label were found in fraction 23. These results indicated that anti-RMC_{abs} reacted with a RBL cell surface molecule having an apparent m.w. similar to that of the receptor for IgE. When ^{131}I-labelled RMC surface antigens precipitated by the cross-reacting anti-RBL_{abs} were analyzed on the same gel with ^{125}I-labelled RBL cell surface components associated with the same antiserum, again peaks of similar mobilities were obtained (Fig. 19b). Similarities appeared in fractions 20 and 24. The above results indicate that both RMC and RBL cells might share common surface antigens found between fractions 18 - 26 on 5% gels. There was a definite discrepancy in the number of peaks of RMC or RBL cell surface antigens resolved by SDS-PAGE of different gel porosities. On 5% gels, only five peaks were seen when RMC surface antigens were reacted with anti-RMC_{abs} (Fig. 3). However, when 10% gels were used for the analysis, three additional peaks were resolved (Fig. 11a). This was also true for the analysis of RBL cell surface antigens. There were

Fig. 18 5% SDS-PAGE Analysis of RMC and RBL Cell Surface Antigens

The extract of RBL cells (1×10^7) labelled with ^{125}I was allowed to react with 0.2 mg of anti-RBL_{abs} at 37°C for 30 mins. A predetermined, optimal amount of goat anti-rabbit immunoglobulin (GAR) was added and the reaction continued for another 30 mins. The immunoprecipitates were allowed to form at 4°C over a period of 3 - 4 hours. The ^{125}I-labelled RBL cell surface antigens reacting with anti-RBL_{abs} (●————●) was then analyzed on the same gel with the precipitates obtained the same way from ^{131}I-labelled RMC surface antigens (2.5×10^6) reacting with 0.2 mg of anti-RMC_{abs} (o- - - - -o).

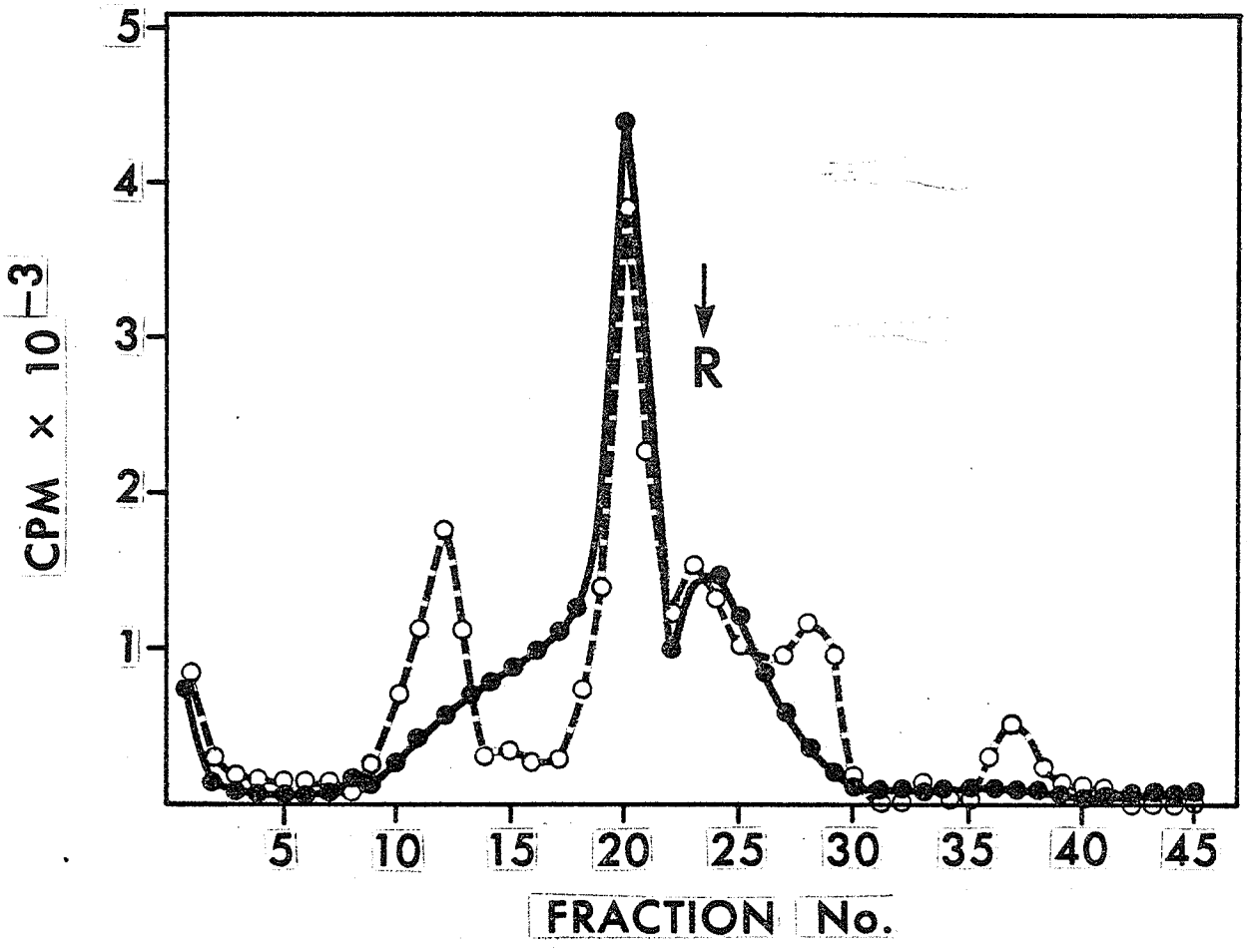
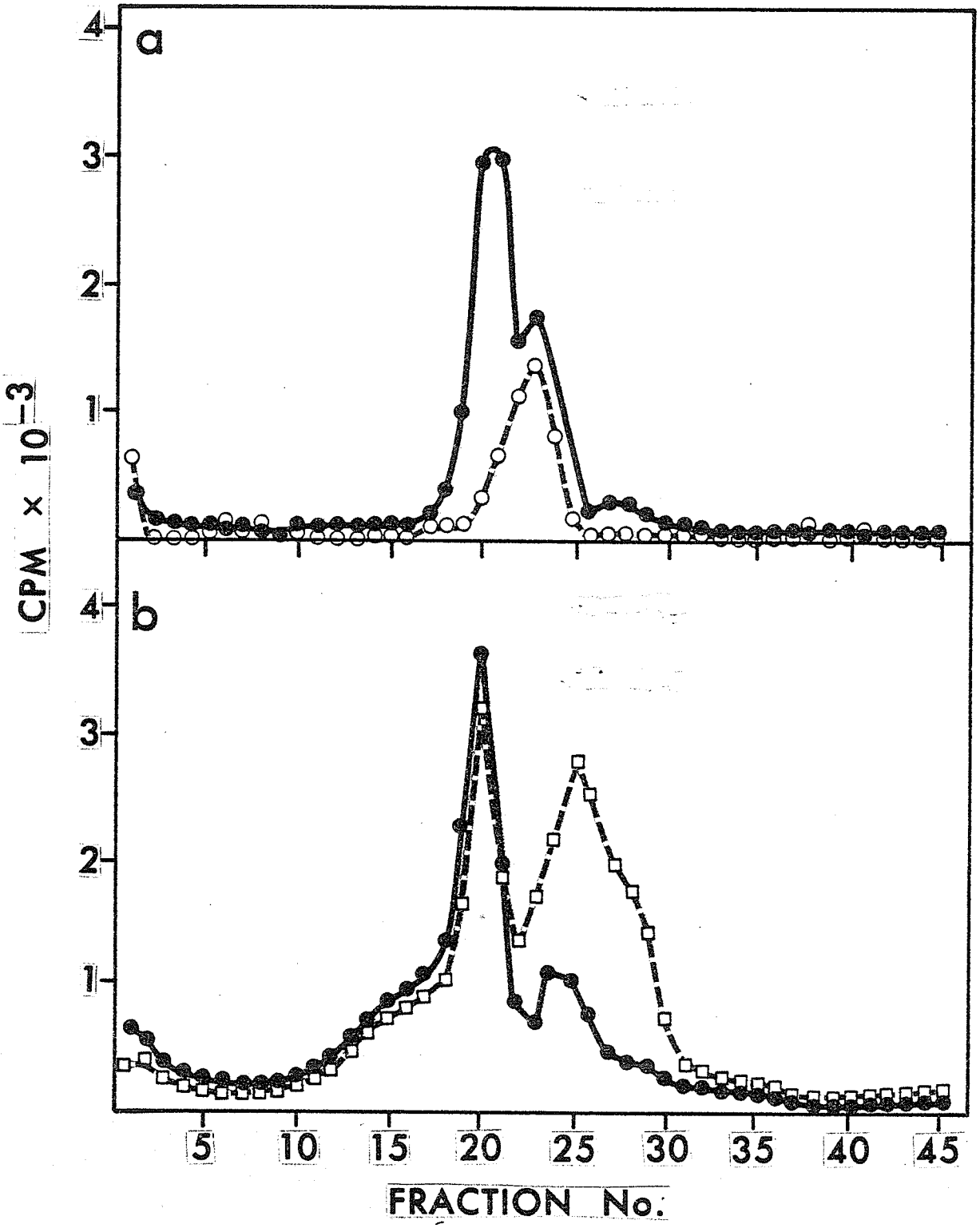

CPM × 10⁻³
5
4
3
2
1
R
5
10
15
20
25
30
35
40
45
FRACTION No.

Fig. 19 5% SDS-PAGE Analysis of Cross-Reacting RMC and RBL Cell Surface Antigens

(a) Immunoprecipitates formed between the extract of ^{125}I-labelled RBL cells (1×10^7), anti-RMC$_{abs}$ (0.2 mg) and GAR (see the legend of Fig. 18) (●——●) were analyzed on the same gel with the surface antigens of ^{131}I-labelled RBL cells (1×10^7) precipitated by IgE (10 µg), rabbit anti-rat IgE (0.2 mg) and GAR (○- - - -○).

(b) Immunoprecipitates obtained from the surface antigens of ^{125}I-labelled RBL cells (1×10^7) reacting with anti-RBL$_{abs}$ (0.2 mg) and GAR (●——●) were analyzed on the same gel with the surface antigens of ^{131}I-labelled RMC (2.5×10^6) precipitated by anti-RBL$_{abs}$ (0.2 mg) and GAR (□- - - -□).


a
b
CPM × 10⁻³
FRACTION No.
1
2
3
4
5
10
15
20
25
30
35
40
45

only two prominent peaks observed in the 5% SDS-PAGE analysis of RBL cell surface components precipitated by anti-RBL_{abs} (Fig. 18) whereas at least seven peaks were revealed by the 10% SDS-PAGE analysis (Fig. 13a). Not only does 10% SDS-PAGE have a better resolving power for proteins of m.w. between 10,000 to 150,000 daltons than 5% SDS-PAGE (Weber and Osborn, 1975), but also longer gels (140 mm versus 90 mm) and a stacking gel with a discontinuous Tris-buffer system (Laemmli, 1970) were used for the analysis. Because of the superiority of the 10% SDS-PAGE analysis, additional comparisons were made using this gel system.

The ^{125}I-labelled RMC surface antigens precipitated by anti-RMC_{abs} were analyzed on the same gel with ^{131}I-labelled RMC surface antigens bound by anti-RBL_{abs}. The results showed that both antisera reacted with similar or identical RMC surface components which appeared in fractions 25 - 40 (m.w. of 30,000 - 60,000 daltons) (Fig. 20a). Similar results were obtained when RBL cell extracts were used instead of RMC surface antigens (Fig. 20b). It was clearly demonstrated that both RMC and RBL cells contained some common surface antigens capable of reacting with both anti-RMC_{abs} and anti-RBL_{abs}. Once again, these common antigens appeared between fractions 25 and 40. In addition, it does appear that the surface molecule migrating near fraction 9 may be common to both cell types. This is suggested by the fact that anti-RBL_{abs} reacted with a component of identical m.w. from either cell type. It should be noted that four peaks, among the rest of RMC surface antigens bound by anti-RMC_{abs}, would usually appear around fractions 3, 5, 9 and 13 (Fig. 11a). These peaks are not distinctly recognizable between fractions 3 and 13 in Fig. 20a. These differences may

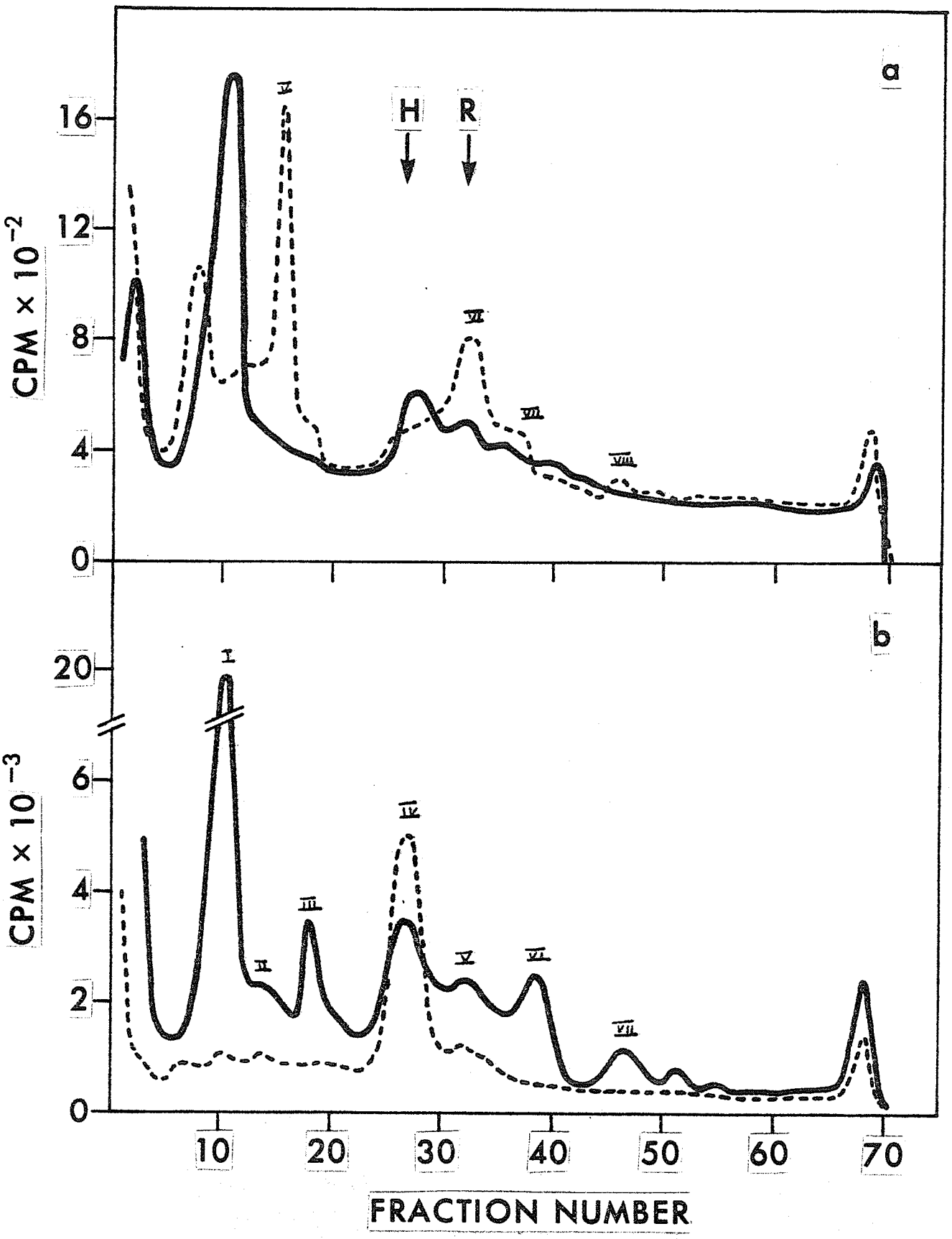
Fig. 20 10% SDS-PAGE Analysis of Cross-Reacting RMC and RBL Cell Surface Antigens

(a) The surface antigens of ^{125}I-labelled RMC (2.5×10^6 cells) reacting with anti-RBL_{abs} (0.4 mg) (————) were analyzed on the same gel with the surface components of ^{131}I-labelled RMC (2.5×10^6 cells) bound by anti-RMC_{abs} (0.4 mg) (- - - - -).

(b) The surface antigens of ^{125}I-labelled RBL cells (1×10^7 cells) bound by anti-RBL_{abs} (0.4 mg) (————) were analyzed on the same gel with the surface components of ^{131}I-labelled RBL cell extract (1×10^7 cells) reacting with anti-RMC_{abs} (0.4 mg) (- - - - -).

The immune complexes were insolubilized by Protein A-Sepharose (0.1 ml per sample) and were eluted with a sample buffer containing 2% SDS prior to the analysis.

H and R represent the positions where the receptors, as defined by Conrad and Froese (1978a), would be expected to migrate.


a
b
H
R
CPM × 10⁻²
CPM × 10⁻³
FRACTION NUMBER
0
4
8
12
16
20
2
6
10
30
40
50
60
70

have been caused by overloading of the gel in the case of dual labelling experiments. Nevertheless, the presence of the other RMC surface antigens precipitated by either anti-RMC_{abs} or anti-RBL_{abs} is still clearly demonstrable. It should also be noted that anti-RBL_{abs} precipitated another component, appearing around fraction 27, of the RMC surface antigens (Fig. 20a). The presence of such a component among the rest of RMC surface antigens bound by anti-RMC_{abs} had never been clearly demonstrated although the possibility of its existence was indicated by the 'shoulder' appearing near the same region (Fig. 11a & 20a). In view of the fact that anti-RMC_{abs} had been able to precipitate a RBL cell surface component of similar mobility (Fig. 20b), it can be concluded that both RMC and RBL cells might share yet another common antigen that appeared around fractions 26 - 27, having an apparent molecular weight of 55,000 daltons (Table VIIb). In addition, it may also suggest that the frequency of occurrence of the 55,000 dalton component is probably lower on the RMC surface than that on the RBL cell surface. By the same token, anti-RBL_{abs} reacted with a RMC surface component appearing in fraction 3 (Fig. 20a) whereas it seemed not to recognize any antigens of the same mobility on the RBL cell surface (Fig. 20b). It is possible that both cell types share the same antigen but may express it differently on their surface. Most other surface molecules which have not yet been mentioned but were precipitated by either anti-RMC_{abs} or anti-RBL_{abs} (Fig. 20) were most likely unique to either cell type. Particularly obvious in the case of RMC are the peaks near fractions 5 and 16. As for RBL cells, a peak in fraction 18 is a good candidate. However, because of the facts that molecules of the same mobility / molecular weight may differ anti-

genically and molecules of different mobility / molecular weight may be antigenically similar, further studies were undertaken to identify common and unique antigens of the two cell types.

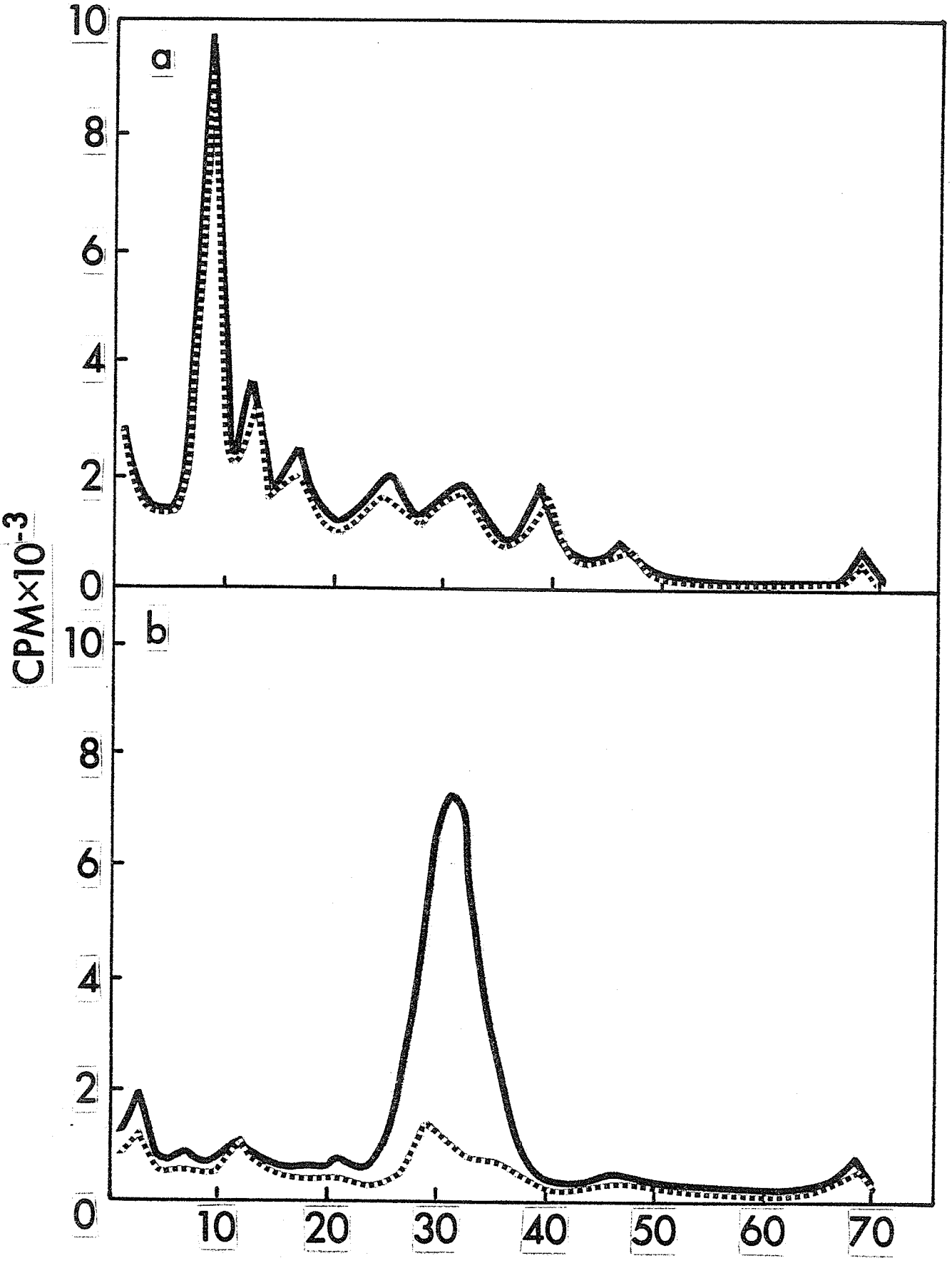
Both anti-RMC_{abs} and anti-RBL_{abs} had been shown to inhibit IgE binding to RMC and RBL cells (Fig. 10). Moreover, the receptors for IgE on RBL cells have been shown to consist of two molecules, H and R with m.w. of 55,000 and 45,000 daltons, respectively (Conrad and Froese, 1978a; Helm et al., 1979). Both molecules were also observed on the surface of RMC, with R appearing to be the more prominent one (Froese, 1979). Both molecules would be expected to migrate to the region encompassing fractions 25 - 35. Therefore, it was expected that the receptor(s) for IgE might be among the common antigens of RMC and RBL cells. To test for the presence of anti-receptor antibodies in anti-RBL_{abs} and anti-RMC_{abs}, several approaches were possible. Since the antisera had been shown to block the binding between IgE and its target cells (Fig. 10), it was hoped that IgE might inhibit the reaction in solution between the receptor and any anti-receptor antibodies present in the antisera. In addition, it had also been shown that IgE bound to the cell surface of either RMC or RBL cells blocks the radioiodination of the receptor (Conrad and Froese, 1976). Therefore, a batch of RBL cells was exposed to excess IgE, washed and subsequently solubilized with NP-40. A control batch of cells was surface labelled in the absence of IgE and was solubilized as described before. Antigens from both cell batches were precipitated by either anti-RBL_{abs} or anti-IgE and were analyzed by SDS-PAGE on 10% gels. Neither the profile nor the intensity of the peaks, originating from cells exposed to excess IgE, differed significantly from that of cells labelled in

the absence of IgE (Fig. 21a). However, the control showed that precipitable receptor cpm were greatly reduced when IgE was present during the labelling of the cells (Fig. 21b). Two possible explanations can be offered for these negative results: (1) the antiserum did not contain any or only small numbers of antibodies to the receptor or (2) only a few of the antigens migrating into the receptor region were indeed receptor molecules. Of course, it can be argued that if both IgE and anti-receptor antibodies react with determinants that are far apart, no inhibition should occur. However, since radioiodination was carried out when receptors were blocked by bound IgE, any receptors associated with the antibodies should not have been revealed in the SDS-PAGE profile. Meanwhile, another test for the presence of anti-receptor antibodies was performed as follows. The receptor molecules were purposely depleted from the pool of the radio-labelled, solubilized surface antigens prior to the exposure to the antisera. The removal of the receptor molecules could be accomplished by either pre-precipitating the receptor using the IgE-anti-IgE system or passing the surface extract through an IgE affinity column as described by Conrad and Froese (1978a). Two batches of RMC were radioiodinated and were solubilized with NP-40. The ^{125}I-labelled RMC extracts were pooled and redivided into four aliquots. Two of the aliquots were reacted with the IgE and anti-IgE followed by adsorption of the immune complexes by Protein A-Sepharose. The other two batches were allowed to react with normal rabbit serum and subsequently with Protein A-Sepharose. The four supernatants were removed and incubated with the appropriate antisera. The surface antigen-antibody complexes were isolated as usual and were analysed separately by 10% SDS-PAGE. The results showed that the profile of the ex-

Fig. 21 10% SDS-PAGE Analysis of RBL Cell Surface Antigens Radioiodinated in the Presence or Absence of IgE

A batch of RBL cells (2×10^7) was incubated with IgE (0.1 mg) at 37°C for 2 hours. After thorough washing, the cells were radioiodinated followed by solubilization with PBS/0.1% NP-40. The cell extract was then divided into 2 aliquots. One aliquot was incubated with anti-RBL_{abs} (0.4 mg) and the other with rabbit anti-rat IgE (0.4 mg) at 37°C for 30 mins. The immune complexes were insolubilized with Protein A-Sepharose and were eluted from the latter with a sample buffer containing 2% SDS. Another batch of RBL cells was radioiodinated in the absence of IgE. The detergent extract was also divided into 2 aliquots which were subsequently reacted with either anti-RBL_{abs} (0.4 mg) or IgE (10 µg) and anti-IgE (0.4 mg). The immune complexes were isolated the same way as above for SDS-PAGE analysis.

RBL cells radioiodinated in the presence of IgE (- - - -)
RBL cells radioiodinated in the absence of IgE (————)
(a) Cell surface antigens precipitated by anti-RBL_{abs}.
(b) Cell surface antigens precipitated by anti-IgE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tract which had been absorbed with NRS and subsequently reacted with anti-RMC_{abs} was very similar to that which had been depleted of receptors (Fig. 22a). Absorption of the extract with IgE and anti-IgE appeared to slightly decrease the cpm in the areas between fractions 7 - 11 and between fractions 20 - 50. The loss of surface molecules in this case was probably due to trapping, rather than a selective removal of molecules. By comparison, the pre-precipitation procedure had more significantly reduced the counts of the receptors from the surface antigens bound by IgE and anti-IgE (Fig. 22b). The results indicated that if receptors for IgE were among the antigens precipitated by anti-RMC_{abs}, they would represent only a small fraction of the total.

In another experiment, absorption of whole RBL cell extracts was performed with IgE-Sepharose while control extract were absorbed with NRS-Sepharose. Aliquots of absorbed extracts were allowed to react with either anti-RBL_{abs}, anti-RMC_{abs} or IgE and anti-IgE. Analysis of the bound surface molecules was, once more, achieved by 10% SDS-PAGE. The results are shown in Fig. 23. As can be seen, the patterns of RBL cell surface antigens precipitated by anti-RBL_{abs} from both IgE-Sepharose and NRS-Sepharose absorbed extracts were very similar (Fig. 23a), and would correspond to profiles seen previously (Figs. 13 & 20). However, the IgE-Sepharose absorbed extract exhibited significantly reduced peaks covered between fractions 22 and 50. Peaks IV and V (fractions 22 - 35) were particularly reduced. This latter area of the gels would also be expected to contain receptors, H and R, if they were present among the RBL cell surface antigens precipitated by anti-RBL_{abs}. Since absorption had indeed removed a significant amount of

Fig. 22 10% SDS-PAGE Analysis of RMC Surface Antigens Pre-precipitated with NRS or IgE and Anti-IgE

Two batches of RMC (5×10^6 cells) were radioiodinated and were solubilized with NP-40. The cell extracts were pooled and redivided into 4 aliquots. Two of the aliquots were precipitated with IgE (10 µg) and rabbit anti-rat IgE (0.4 mg) while the other two were reacted with NRS (0.4 mg). All four samples were then incubated with Protein A-Sepharose (0.1 ml each) at 4°C for 3 hours. The supernatants were removed and were reacted with either anti-RMC_{abs} (0.4 mg) or IgE (10 µg) and anti-IgE (0.4 mg) at 37°C for a period of one hour. The immune complexes were insolubilized with Protein A-Sepharose and eluted from the latter for subsequent analysis.

Cell surface antigens pre-precipitated with NRS (————)

Cell surface antigens pre-precipitated with IgE and anti-IgE (- - - - -)

(a) RMC surface antigens reacting with anti-RMC_{abs}

(b) RMC surface antigens reacting with IgE and anti-IgE

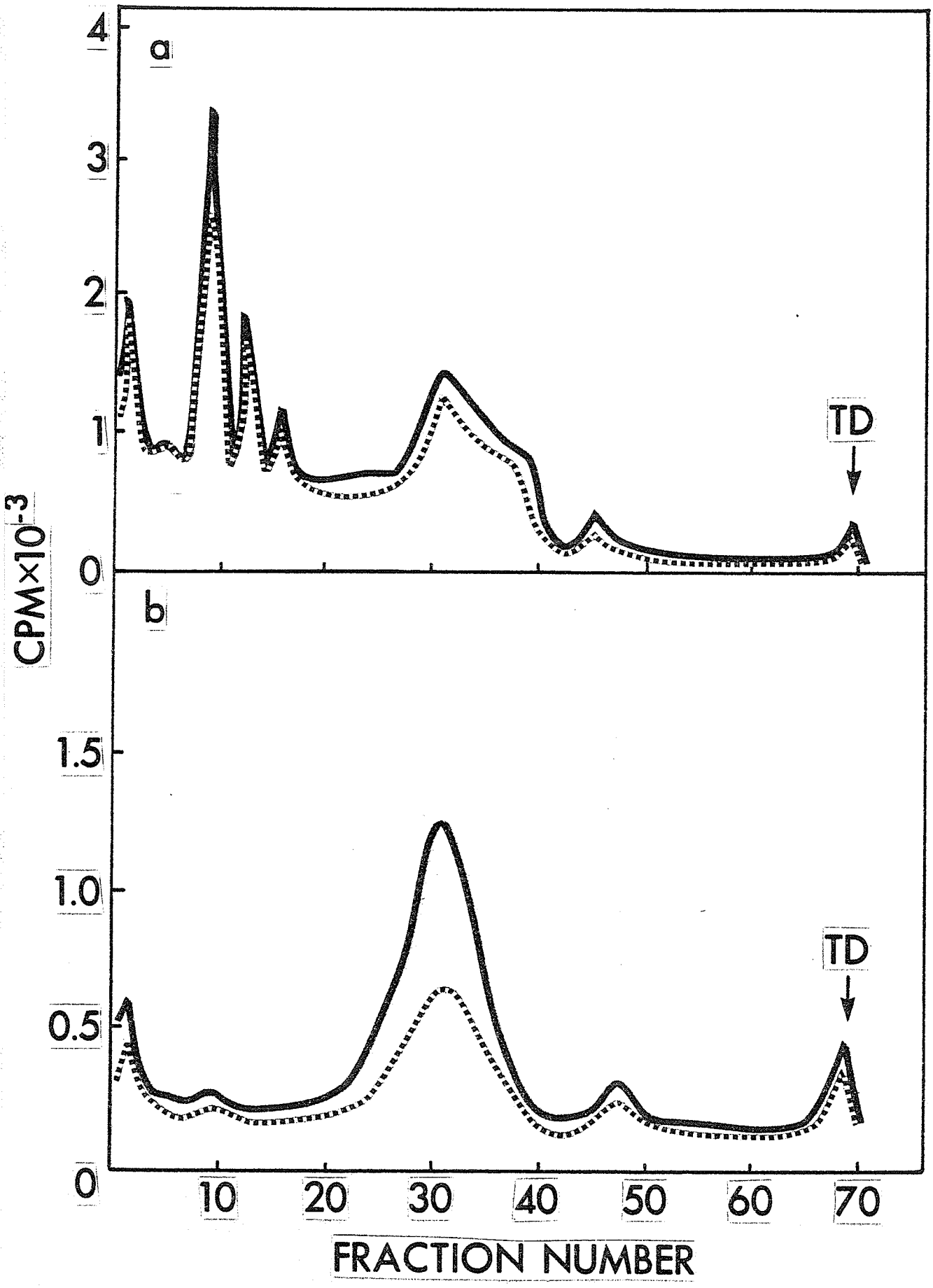
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Fig. 23 10% SDS-PAGE Analysis of RBL Cell Surface Antigens Absorbed with either NRS-Sepharose or IgE-Sepharose

Two batches of RBL cells (3×10^7 cells per batch) were radioiodinated and solubilized as described before. The cell extracts were pooled and redivided into two aliquots. One aliquot was absorbed with NRS-Sepharose in a volume ratio of 1:1 and the other one was absorbed with IgE-Sepharose at the same ratio. All absorptions were carried out at $4^{\circ}C$ for one hour. The supernatants were removed and each was redivided into 3 aliquots which were reacted with either anti-RBL_{abs} (0.4 mg), anti-RMC_{abs} (0.4 mg) or IgE (10 µg) and anti-IgE (0.4 mg). The immune complexes were insolubilized with Protein A-Sepharose and were eluted from the latter for subsequent analysis.

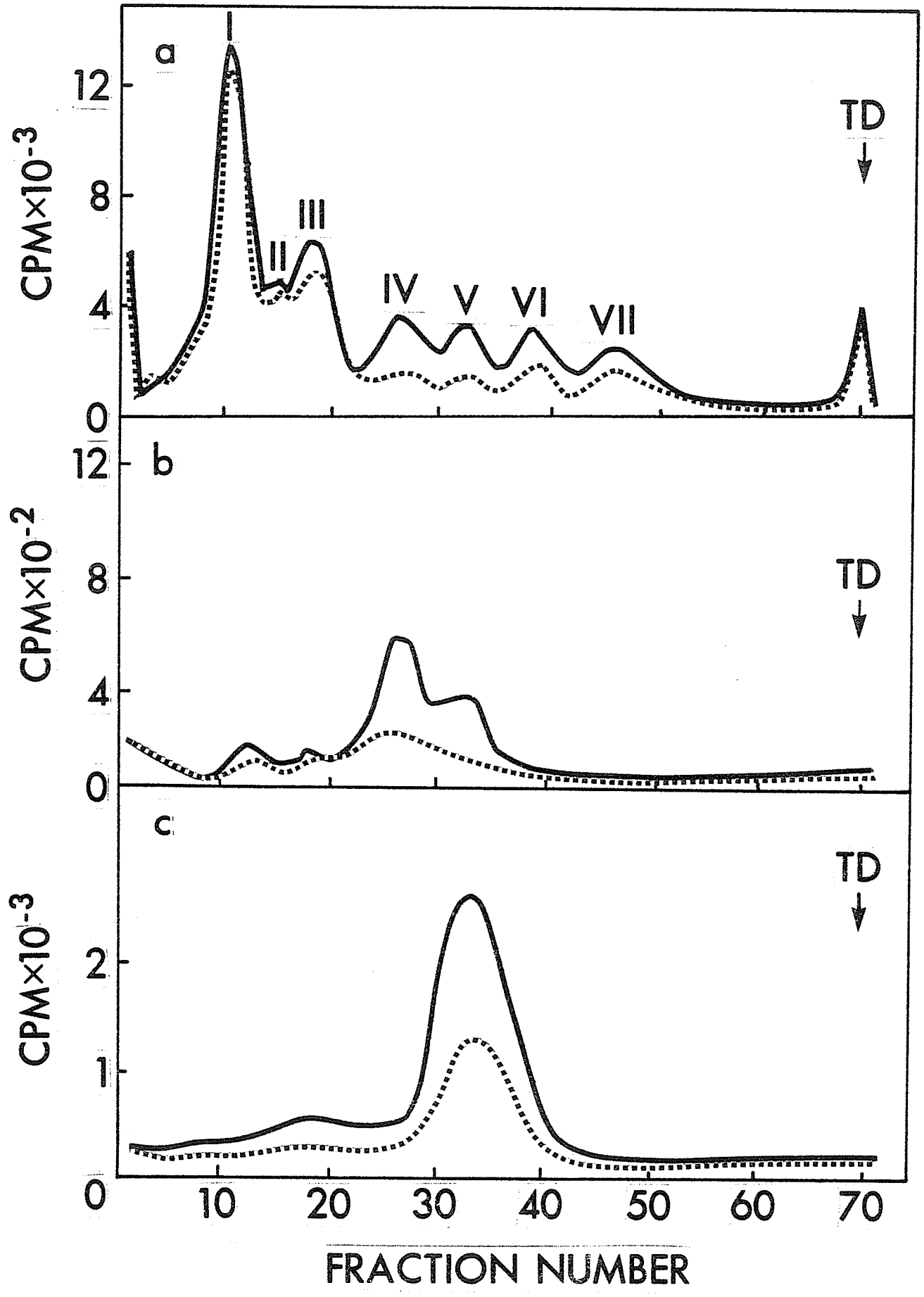
RBL cell surface antigens absorbed with NRS-Sepharose (———)

RBL cell surface antigens absorbed with IgE-Sepharose (- - - -)

(a) Cell surface antigens reacting with anti-RBL_{abs}

(b) Cell surface antigens reacting with anti-RMC_{abs}

(c) Cell surface antigens reacting with IgE and anti-IgE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the R component (Fig. 23c), it is tempting to conclude that the reduction of peaks IV and V (Fig. 23a) was due to the removal of H and R components and that, therefore, anti-RBL_{abs} contained antibodies specific for these two receptors. It is also particularly interesting to see that the two major surface components of RBL cells precipitated by anti-RMC_{abs} were drastically reduced by absorption with IgE-Sepharose (Fig. 23b). These results do suggest that anti-RMC_{abs} cross-reacts mainly with H and R components of RBL cells. Indeed, any antibodies to the receptors for IgE, if present in either anti-RMC_{abs} or anti-RBL_{abs}, would be expected to cross-react with the corresponding receptors of the other cell type. It had been shown previously that receptors of both cell types are very similar in terms of their affinity for IgE (Conrad et al., 1975) and their molecular weight (Conrad and Froese, 1976; Froese, 1979). Moreover, the mobilities of the peaks observed with NRS-Sepharose absorbed extracts (Fig. 23b) are very similar to those observed for H and R components (Conrad and Froese, 1978a). The fact that anti-RMC_{abs} contained antibodies to H and R will be shown more directly later.

At present, it is not quite clear why absorption with IgE-Sepharose appeared to demonstrate the presence of anti-receptor anibodies in the antisera, while absorption with IgE and anti-IgE failed to do so. However, two possible explanations may be offered. (1) The absorption with IgE-Sepharose was more effective in removing receptor than IgE and anti-IgE. The suggestion that this was the case can be seen in Fig. 23c, which shows that about 53.9% of the R components had been removed by the absorption whereas about 46.5% of the same molecules were absorbed as in Fig. 22b. These percentages were calculated from plani-

metric measurements after subtraction of background cpm. (2) More importantly, IgE-Sepharose would be expected to remove both H and R components from the cell extract while IgE and anti-IgE would be expected to absorb only R components. Partial removal of two neighbouring components would be more noticeable on SDS-PAGE patterns than the removal of one.

The absorption experiments with IgE-Sepharose had suggested that both anti-RMC_{abs} and anti-RBL_{abs} may have contained anti-receptor antibodies. The subsequent experiments were, therefore, undertaken to demonstrate the presence of these antibodies more directly. Conrad and Froese (1978a) had reported the isolation of free receptors for IgE either via an IgE-affinity column or in the form of IgE-receptor complexes through the use of DNP-IgE and anti-DNP antibodies (1978b). Both approaches were used in the present study. Isolated receptors or IgE-receptor complexes were subsequently allowed to interact with either anti-RMC_{abs} or anti-RBL_{abs}. Receptor isolation was achieved by means of IgE-Sepharose as described under METHODS, using the procedure of Conrad and Froese (1978a). As can be seen in Table VIII, this method, which isolated both H and R receptors yielded about 8% of the total ^{125}I-labelled RBL cell surface components. In control experiments with NRS-Sepharose only 3% of the surface materials were isolated. Isolation of IgE-receptor complexes, involving primarily the R component, was carried out with ^{131}I-labelled $DNP_{7.7}$-IgE and bovine anti-DNP antibodies coupled to Sepharose (see METHODS). Only about 2% of the cell surface cpm were isolated by this technique. The amount of cell bound DNP-IgE isolated was about 40% (Table VIII). These yields were somewhat lower than those reported by Conrad and Froese (1978b) while those obtained

TABLE VIII

ISOLATION OF RECEPTORS FOR IgE BY MEANS OF AFFINITY CHROMATOGRAPHY[a]

Cell Type	Protein-Sepharose Conjugate	Eluting Agent	% of Cell Surface cpm Bound and Eluted
RBL	IgE	KSCN	8.0 - 8.8
RBL	NRS	KSCN	2.8 - 3.0
RBL	Anti-DNP	DNP-ONa	1.7 - 2 (38 - 46)[b]
RMC	Anti-DNP	DNP-ONa	1.7 (40)[b]

a. Cells were labelled with ^{125}I by the lactoperoxidase procedure.

b. Numbers in brackets indicate the percentage of ^{131}I-IgE eluted in the form of IgE-receptor complexes.

with IgE-Sepharose were about the same as those observed by these authors (1978a).

Isolated-receptor preparations were subjected to the following experiments. After dialysis against the sample buffer to remove KSCN, the eluate from the IgE-Sepharose which had been incubated with the extract from two batches of radioiodinated RBL cells was divided into several aliquots. Enough SDS was added to one of the aliquots to make a final concentration of 2% SDS in solution. The other aliquots were allowed to react with different reagents. When antisera were used, insolubilization of the antigen-antibody complexes was achieved by means of Protein A-Sepharose. Bound materials were eluted with the sample buffer containing 2% SDS and were analyzed by SDS-PAGE on 10% gels. The RBL cell surface antigens isolated through IgE-Sepharose were composed of two components that appeared between fractions 23 and 37 (Fig. 24a). The result was in agreement with the data obtained by Conrad and Froese (1978a) who had demonstrated that, by means of this technique, two receptors for IgE having apparent m.w. of 55,000 and 45,000 daltons can be isolated. These components were later designated as H and R components, respectively (Helm et al., 1979). When the eluate from IgE-Sepharose was precipitated with IgE and anti-IgE, only one major component appearing with a maximum in fraction 33 was observed. This finding was in agreement with the observation of Conrad and Froese (1978a) who found that by means of IgE-anti-IgE, mainly the R component was isolated. On the other hand, both anti-RMC_{abs} and anti-RBL_{abs} precipitated H and R components (Figs. 24c & 24d). Both antisera reacted with more H than R components. This is of particular interest in the case of anti-RMC_{abs} since RMC appear to carry relatively little

Fig. 24 10% SDS-PAGE Analysis of KSCN Eluates

Two batches of RBL cells (2×10^7 cells per batch) were radioiodinated and solubilized as described before. The cell extracts were pooled together in a total volume of 1.0 ml and were absorbed with 1.0 ml of IgE-Sepharose at $4^{\circ}C$ for 1 hour. After washing the Sepharose with 20 ml of PBS/0.1% NP-40, the bound materials were eluted from the mini-column containing IgE-Sepharose with 3 M KSCN in PBS/0.1% NP-40. The KSCN eluate was then divided into 5 aliquots. The first aliquot was dialysed against 0.0625 M Tris buffer (pH 6.8) at $4^{\circ}C$ overnight. The other four aliquots were dialyzed against PBS/0.1% NP-40 and were subsequently reacted with different reagents. The immune complexes were insolubilized with Protein A-Sepharose and were eluted from the latter with the buffer mentioned above. Sufficient SDS to yield a 2% SDS solution was added to each sample and after heating at $100^{\circ}C$ for 90 seconds, the samples were analyzed.

(a) The KSCN eluate alone

The KSCN eluate precipitated by:

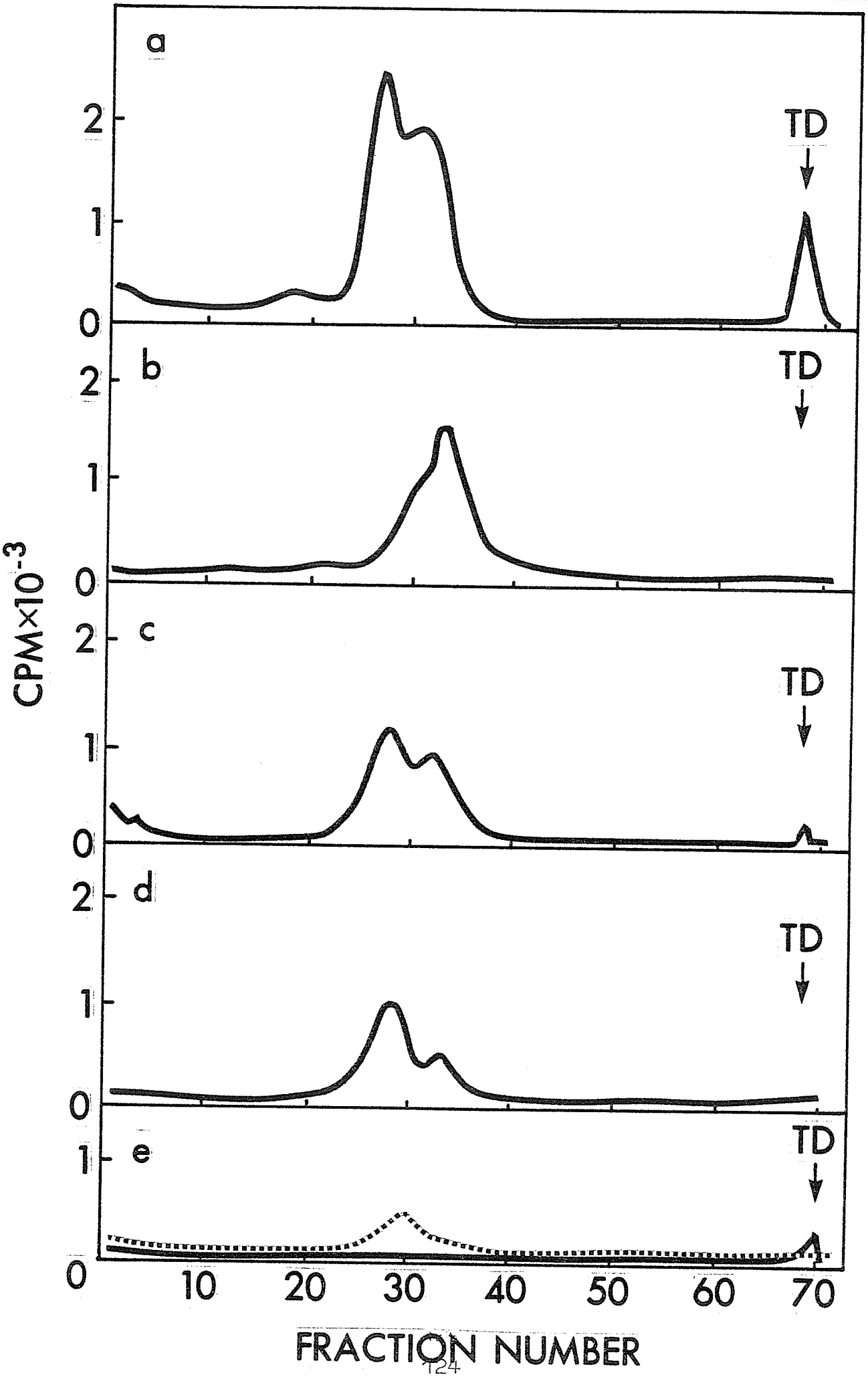
(b) IgE and horse anti-rat IgE

(c) anti-RBL_{abs}

(d) anti-RMC_{abs}

(e) NRS (- - - -) or Protein A-Sepharose (————)

All immunoglobulins used were of the same concentration at 0.4 mg per sample.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of the H component (Froese, 1979). This may mean that the H component is more immunogenic than the R component. As can be seen in Fig. 24e, there was also some binding by NRS. Such binding was most likely part of the property of NRS rather than Protein A-Sepharose since the latter did not bind any of the two receptors for IgE. It is conceivable that this binding was due to cross-reactivity of the receptors for IgE with some of the Fc moieties of the immunoglobulins present. However, the binding of H and R components by immunoglobulins of NRS was much less pronounced than that by the antisera. Rebinding of IgE-Sepharose purified receptors was also tested with anti-RBL coupled to Sepharose. This rebinding was compared to that by IgE-Sepharose. Patterns obtained from SDS-PAGE analysis of the labelled surface antigens bound and eluted from the appropriate gels are shown in Fig. 25. The results again confirmed the capacity of anti-RBL to bind H and R receptors for IgE. As expected, IgE-Sepharose also rebound both components but only to the extent of about 30%, suggesting some denaturation of the receptors upon purification by means of IgE-Sepharose. A similar conclusion was made by Conrad and Froese (1978a) when they first described this method of receptor isolation. It should be mentioned that because of the greater ease of preparing receptors from RBL cells rather than from RMC, the former cell type was primarily used for this purpose. Also because of a limited supply of the anti-RMC serum, fewer studies were carried out with it.

Having established that both antisera did bind the receptors (H and R) purified by means of IgE-Sepharose, the next step was to investigate if they reacted with IgE-receptor complexes. Conrad and Froese (1978b) reported that isolating IgE-receptor complexes via DNP-IgE

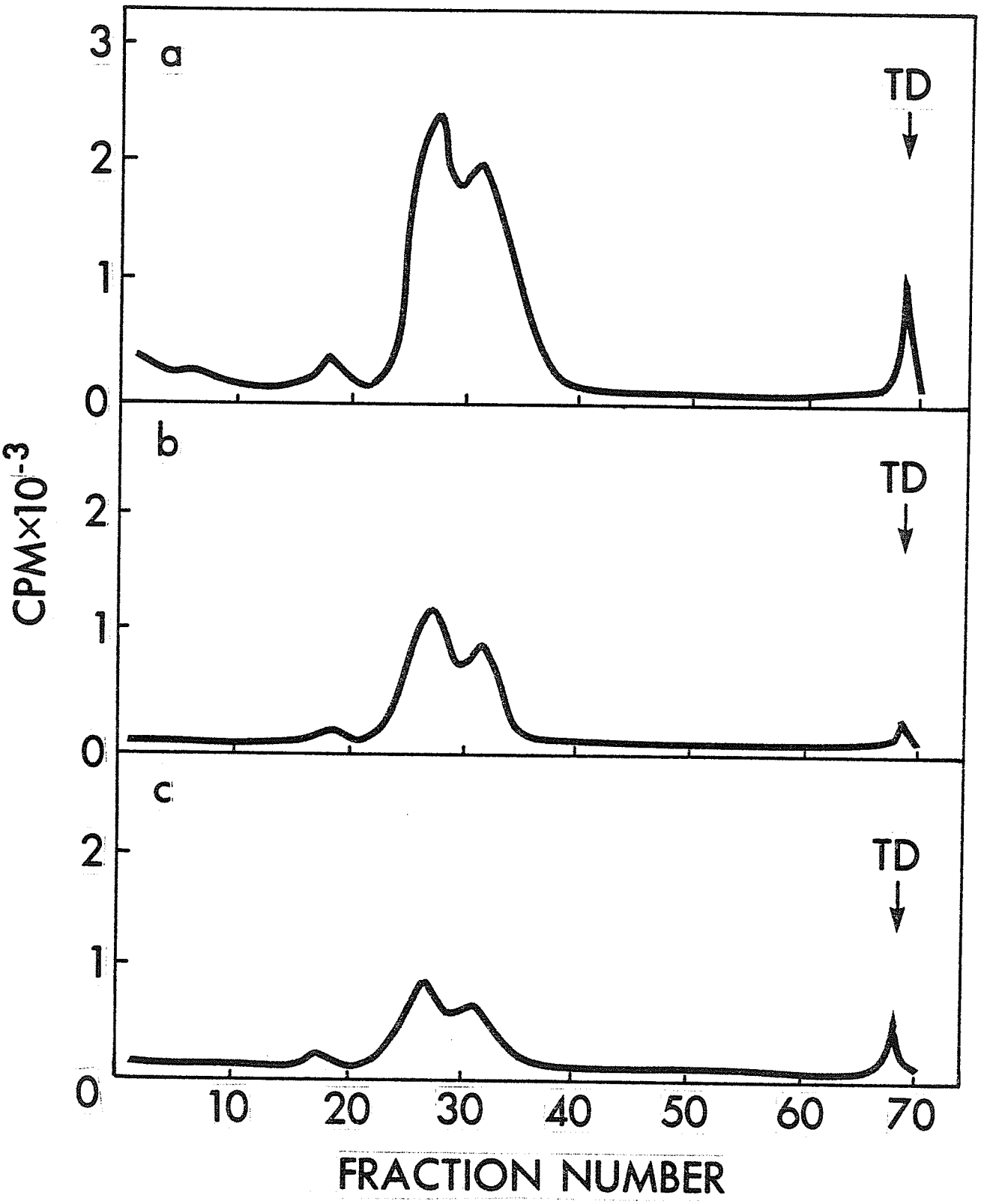
Fig. 25 10% SDS-PAGE Analysis of KSCN Eluates Rebinding to Different Sepharose Conjugates

The KSCN eluates were obtained the same way as described in the legend of Fig. 24.

(a) The KSCN eluate alone

(b) The KSCN eluate (in 0.5 ml) was incubated with 0.5 ml of packed IgE-Sepharose at 4°C for one hour. After thorough washing with PBS/0.1% NP-40, the bound materials were eluted from the absorbent with the sample buffer containing 2% SDS at 100°C for 90 seconds and were subsequently analyzed.

(c) The KSCN eluate (in 0.5 ml) was incubated with 0.5 ml of packed anti-RBL coupled Sepharose. The bound materials were isolated and analyzed the same way as described above.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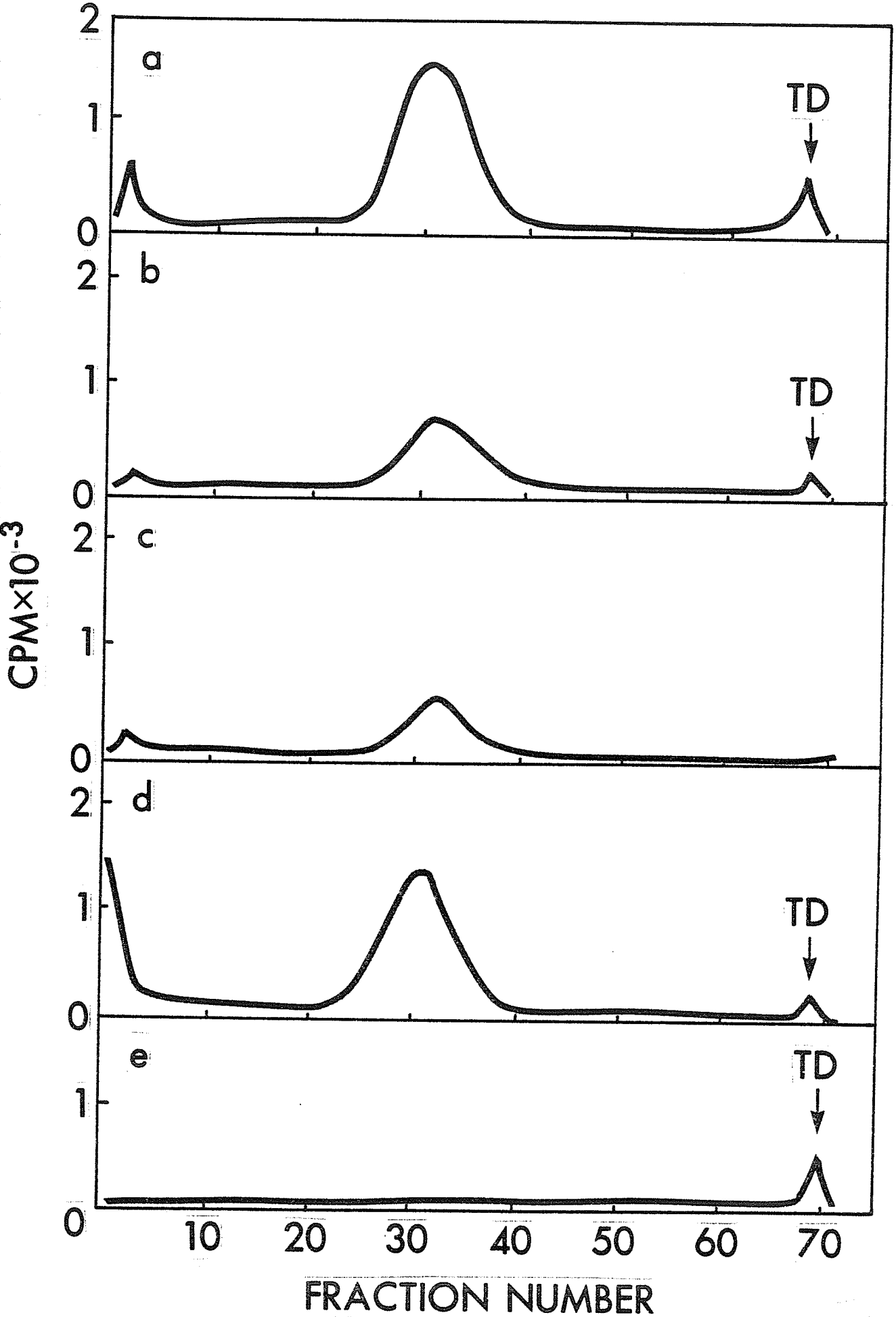
and anti-DNP-Sepharose yielded only one component (R) with an apparent m.w. of 45,000 daltons. Therefore, it was thought to be interesting to find out if either of the two antisera did react with R in the form of IgE-receptor complexes. Hence, IgE-receptor complexes from RMC or RBL cells were obtained as described under METHODS. The bound surface extracts were eluted with a buffer containing dinitrophenolate (DNP-ONa) and the eluate will be referred to as the DNP-ONa eluate. The IgE-receptor complexes from RMC were allowed to react with anti-RMC_{abs} and anti-RBL_{abs} followed by insolubilization with Protein A-Sepharose. The bound materials were then eluted and analyzed by SDS-PAGE. In both instances, the above surface antigens migrated with a mobility resembling that of the R component (Figs. 26b & 26c). In other words, both antisera were capable of binding IgE-receptor complexes. A control using NRS did not bind the complexes (Fig. 26e) while anti-IgE reacted with them (Fig. 26d). These results further confirm that the R component was among the common antigens present on the two cell types. Almost identical results were obtained when RBL cells were used as the source of IgE-receptor complexes. Once again, both antisera were shown to react with the complexes while NRS did not bind any of them (Fig. 27). The fact that both antisera were capable of reacting with intact IgE-receptor complexes is illustrated in Table IX. Hence, both anti-RMC_{abs} and anti-RBL_{abs} could precipitate receptor (^{125}I-cpm) as well as DNP-IgE (^{131}I-cpm). The finding that both antisera bound a higher percentage of receptor cpm than IgE cpm does suggest that some dissociation of the complexes had taken place after elution with DNP-ONa. Had this not been the case, identical percentages of cpm should have been bound. The fact that anti-IgE reacted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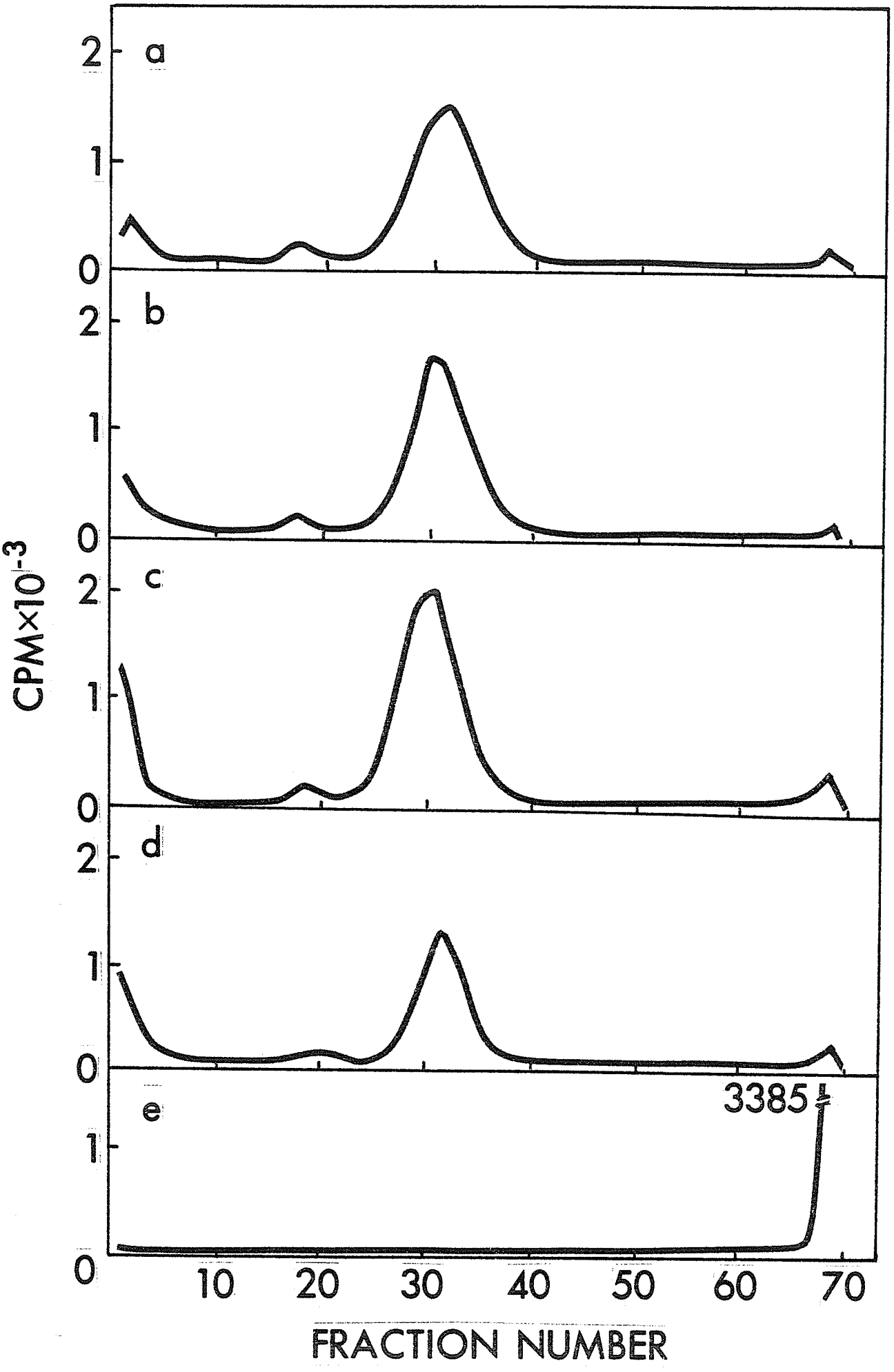
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TABLE IX

BINDING OF IgE-RECEPTOR COMPLEXES BY DIFFERENT SERA[a]

Cell Type	Serum	% Receptor Bound (^{125}I)	% IgE Bound (^{131}I)
RMC	anti-IgE	33.3	55.5
	anti-RMC_{abs}	20.0	11.7
	anti-RBL_{abs}	14.0	10.0
	NRS	2.0	0
RBL Cells	anti-IgE	32.0	60.0
	anti-RBL_{abs}	21.4	18.0
	anti-RMC_{abs}	17.0	16.0
	NRS	1.8	0

a. Cells were radioiodinated and solubilized and the IgE-receptor complexes were isolated as described under METHODS. Immune complexes were insolubilized with Protein A-Sepharose and after thorough washing they were counted for their radioactive contents. The percentage of ^{131}I-IgE / ^{125}I-receptor bound was calculated from the following formula:

$$\% \text{ bound} = \frac{\text{cpm of bound materials}}{\text{cpm of IgE-receptor}} \times 100\%$$

with a higher percentage of IgE than surface antigens is indicative of some dissociation of the complexes. It is also conceivable that anti-IgE which was used in excess over IgE had a higher capacity to react with the complexes than either anti-RMC_{abs} or anti-RBL_{abs}. Again, NRS did not significantly react with the complexes (Table IX).

It is now certain that both anti-RMC_{abs} and anti-RBL_{abs} did contain antibodies to common surface antigens shared by RMC and RBL cells. Two of these common antigens had been identified as the receptors for IgE. Data from analytical electrophoresis revealed that the apparent m.w. for both components were 55,000 and 45,000 daltons, respectively, which agree with the results of Conrad and Froese (1978a). There were at least three more surface antigens that were found present on both cell types, namely, peaks III, VII and VIII of the RMC surface (Table VIIa) and peaks I, VI and VII of the RBL cell surface (Table VIIb). For convenience, their similarity in terms of apparent m.w. will be simplified as follows: peak III_{RMC} = peak I_{RBL}; peak VII_{RMC} = peak VI_{RBL} and peak $VIII_{RMC}$ = peak VII_{RBL}. Additional evidence that they could be the common antigens was given by the fact that anti-RBL_{abs} reacted to all of them (Figs. 20a & 20b). However, anti-RMC_{abs} only reacted with the 55,000 and 45,000 dalton components of RBL cells (Fig. 20b) indicating that it may contain fewer numbers of antibodies to the three antigens.

C. Characterization of RMC and RBL Cell-Specific Antigens:

One of the best ways to find out if both RMC and RBL cells share common surface antigens is to absorb their specific antisera with the other cell type. Such absorption will not only remove antibodies to the common antigens but will also leave relatively pure antibodies

that would react with the specific antigens of each cell type. Hence, anti-RBL was absorbed with RMC (1×10^7 cells per absorption) five times and anti-RMC with RBL cells ($1 - 2 \times 10^8$ cells per absorption) three times as described under METHODS. The reason for using fewer numbers of RMC for the absorption was due to the fact that the RMC purification procedure was too tedious and costly. Only $1 - 2 \times 10^6$ RMC can be obtained from a single rat. The absorbed antisera in this case will be referred to as anti-RBL_{mas} and anti-RMC_{bas}, respectively, for easy identification. Both antisera were then subjected to the following tests. They were first tested for their ability to induce the skin reaction as described in the previous chapter. Next, their effect on inhibiting IgE binding to target cells was also assessed. The results are shown in Tables Xa and Xb. Absorption had reduced the ability of anti-RMC_{bas} to induce the skin reaction, from an antibody titre of 200 to 100. It also abolished the inhibitory effect of the anti-RMC serum on IgE binding to RBL cells. Similarly, both of the activities of the anti-RBL serum had been significantly reduced by the absorption. Since anti-RBL had been absorbed with RMC at a much lower concentration, it was thought that removal of the antibodies to the common antigens may not have been as effective as in the case of anti-RMC_{bas}. Hence, it was not unexpected to find that anti-RBL_{mas} still induced some skin reaction and inhibited IgE binding to RBL cells, although at a much lower capacity.

It was of utmost interest to find out exactly which of the cell surface antigens known already would react with the above antisera. Hence, the surface antigens of either RMC or RBL cells were allowed to react with either anti-RMC_{bas} or anti-RBL_{mas}. The antigen-antibody

TABLE Xa

SKIN REACTIONS INDUCED BY ANTI-RMC_{bas} AND ANTI-RBL_{mas}[a]

Serum \ Dilution	Diameter of Spot (mm)			Color Intensity
	1 : 10	1 : 100	1 : 200	
Anti-RMC_{bas}	10.0	7.0	-	+++
Anti-RMC_{abs}	13.2	8.6	7	++++
Anti-RBL_{mas}	5.0	-	-	+
Anti-RBL_{abs}	11.0	6.2	5	+++
PBS				-

TABLE Xb

INHIBITION OF IgE BINDING TO RBL CELLS BY ANTI-RMC_{bas} AND ANTI-RBL_{mas}[b]

Serum	% of Inhibition
Anti-RMC_{bas}	0
Anti-RMC_{abs}	56
Anti-RBL_{mas}	15
Anti-RBL_{abs}	70
THM/BSA	0

a. Experimental conditions: See the footnote to Table V.

b. About 0.4 mg of rabbit immunoglobulins were incubated with 1×10^6 RBL cells at 37°C for 30 mins. The incubation was continued for another 30 mins after the addition of 0.1 µg of ^{125}I-IgE to each sample. After thorough washing, the cell pellets were counted for their radioactive contents and the results were calculated.

complexes were insolubilized with Protein A-Sepharose as it has been described and were eluted from the latter for SDS-PAGE analysis on 10% gels. The results are shown in Fig. 28. Absorption of anti-RMC had removed most of the antibodies to certain RMC surface antigens that would otherwise appear between fractions 20 and 50. Antibodies to peaks II and IV of RMC surface antigens had also been absorbed by RBL cells (Fig. 28a). The anti-RMC_{bas} serum reacted with only three of the eight RMC surface antigens already defined by anti-RMC_{abs} (Fig. 11a). These were components that appeared as peaks I, III and V (Fig. 28a) with apparent m.w. of 175,000, 130,000, and 85,000 daltons, respectively. They were most likely the specific antigens of RMC.

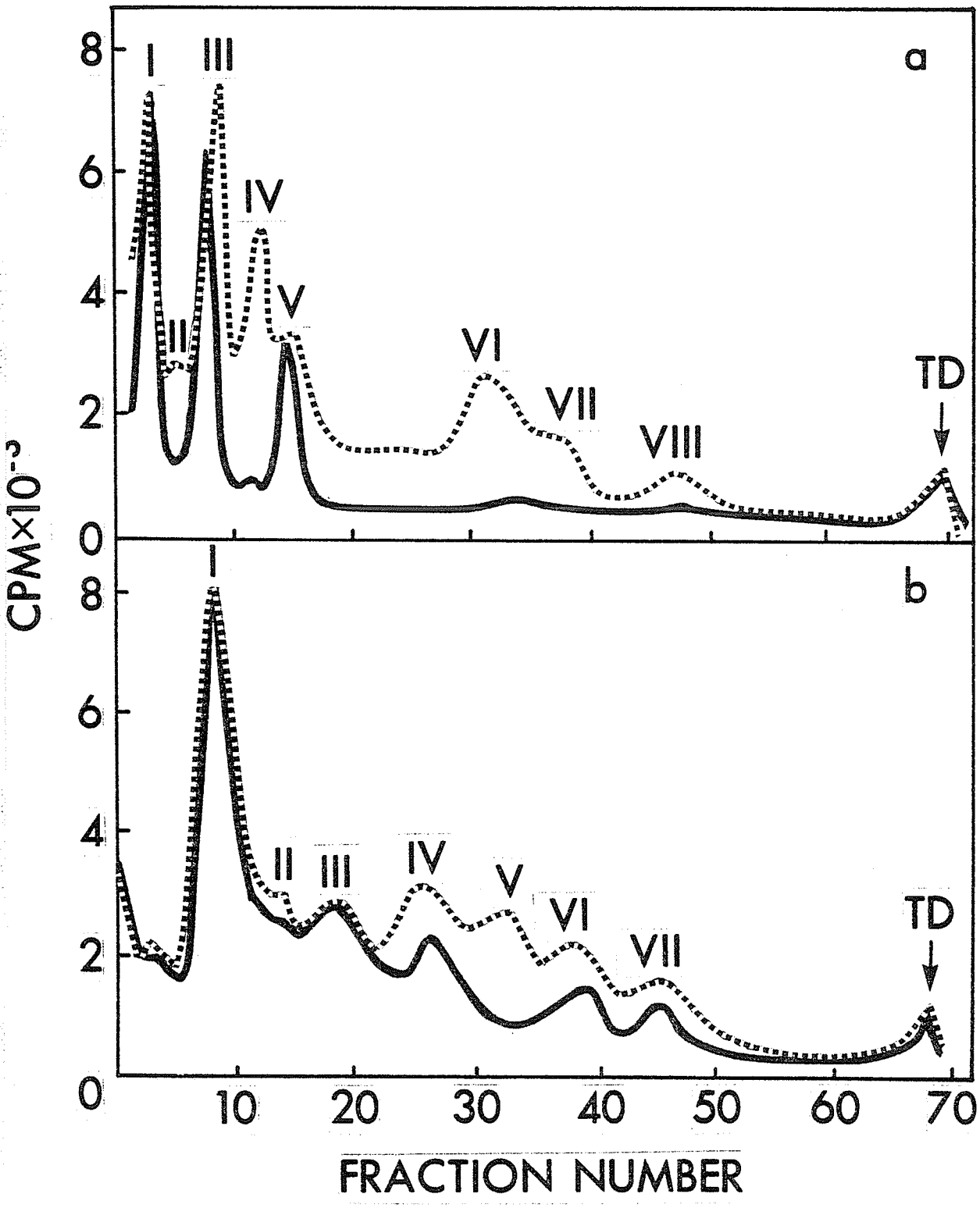
Analysis of the RBL cell surface antigens reacting with anti-RBL_{mas} had revealed results similar to the ones just mentioned. Absorption had removed antibodies to several RBL cell surface antigens appearing between fractions 20 and 50 (Fig. 28b). The complete removal of peak V_{RBL} suggested that the absorption had been effective. On the other hand, the partial reduction of the intensity of peaks II, IV, VI and VII of RBL cell surface antigens indicated that some of the antibodies were still present. This, in turn, would suggest that not all surface antigens of RMC were equally expressed. The antigen with mobility similar to peak V_{RBL} was also present on the surface of RMC. Indeed, it appeared to be the predominant one (Fig. 11). The fact that there were relatively fewer antibodies to peak V than to the others in this area of the gel could also account for the above observation. Data presented in the previous section have clearly demonstrated that the two peaks appearing in fractions 26 and 32 were in fact associated with the H and R receptors for IgE. Both were among the common antigens

Fig. 28 10% SDS-PAGE Analysis of RMC/RBL Cell Surface Antigens Reacting to Anti-RMC_{bas}/Anti-RBL_{mas}

(a) RMC (5×10^6 cells) were radioiodinated and solubilized as described previously. The cell extract was divided into two aliquots. One aliquot was incubated with 0.4 mg of anti-RMC_{abs} (- - - -) and the other with anti-RMC_{bas} (————) of the same amount at 37°C for 30 mins.

(b) RBL cells (2×10^7 cells) were surface labelled and solubilized as described before. The cell extract was divided into two aliquots. One aliquot was incubated with 0.4 mg of anti-RBL_{abs} (- - - -) and the other with 0.4 mg of anti-RBL_{mas} (————) at 37°C for 30 mins.

All immune complexes were insolubilized with Protein A-Sepharose. The bound materials were eluted and analyzed as usual.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shared by RMC and RBL cells and precipitated by both anti-RMC_{abs} and anti-RBL_{abs}. It has been demonstrated that H is not a major receptor for IgE present on the RMC surface (Froese, 1979) and that both of the two antisera described in this thesis contained more antibodies to H than to R. Therefore, it is not surprising to see that the intensity of peak IV_{RBL} was only partially reduced. Similar arguments may be invoked to explain why antibodies to the other common antigens were not completely removed. It is, of course, also conceivable that antibodies to surface molecules represented by peaks II, IV, VI and VII were not completely removed because each peak contained more than a single antigen, some of which were not present on RMC. However, the absorption did not affect the profile nor the intensity of only two peaks, I and III of the RBL cell surface antigens (Fig. 28b). Hence, it is plausible that they may represent the unique RBL cell surface antigens with apparent m.w. of 130,000 and 78,000 daltons, respectively. It should be pointed out that when discussing relative amounts of various surface antigens, the assumption is made that all iodinated cell surface molecules have the same specific activity. However, this may not always be correct.

Data from the above absorption study revealed that there were at least six common antigens shared by both RMC and RBL cells. While some of them were readily recognized by both anti-RMC_{abs} and anti-RBL_{abs}, namely peaks $IV_{RMC} = II_{RBL}$, $VI_{RMC} = V_{RBL}$, $VII_{RMC} = VI_{RBL}$ and $VIII_{RMC} = VII_{RBL}$, peaks II_{RMC} and IV_{RBL} were precipitated by only one or the other antiserum (Figs. 28a & 28b). The latter observation indicated that even though both antigens were present on the two cell surface they might still be different in terms of immunogenicity or frequency of occurrence.

Anti-RBL_{abs} seemed to contain very few antibodies to the RBL cell surface antigen having similar mobility as peak II_{RMC}. However, its presence on the RBL cell surface was demonstrated by the removal of the corresponding antibodies from anti-RMC_{abs} upon absorption with RBL cells. Peaks VI_{RMC} (or V_{RBL}) and IV_{RBL} had been shown to have similar mobilities as R and H receptors for IgE, respectively (Figs. 19 & 20). In fact, surface components precipitated by both antisera from the preparations containing free receptors or IgE-receptor complexes were shown to migrate to the same regions of the gel where both H and R appeared (Figs. 24,26 & 27). These results clearly demonstrated the presence of antibodies to H in anti-RMC_{abs} even though such a molecule was not distinctly exhibited in the SDS-PAGE pattern of RMC surface antigens bound by the same antiserum (Fig. 11a). However, this is not surprising since Froese (1979) had demonstrated that RMC may carry only low numbers of H receptors. The fact that most of the antibodies to the above common antigens could be removed by absorption with intact cells suggests that these are antigens that are exposed on the outer layer of the plasma membrane of both RMC and RBL cells.

There were at least three surface antigens of RMC and more than two antigens of RBL cells precipitated by anti-RMC_{bas} and anti-RBL_{mas}, respectively. It is interesting to note that most of these antigens appeared in the high molecular weight region of the gels when subjected to SDS-PAGE analysis. The 130,000 dalton component of RMC surface antigens seemed to be antigenically distinct from the other 130,000 dalton component present on the RBL cell surface. Since anti-RBL_{abs} had been shown previously to react with a RMC surface antigen appearing in about the same fraction (Fig. 20a), it appears that it may have two dif-

ferent molecules of the same or very similar m.w. present in the area. On the other hand, some common antigenic determinants of this component may be buried in the plasma membrane. Thus, the corresponding antibodies cannot be removed by the absorption with intact cells. Therefore, it is conceivable that the 130,000 dalton components of both cell types are in fact identical. However, it is also possible that the area may contain common as well as discrete surface antigens of the two cell types. Further discussion of this component will come later. The partial absorption of antibodies to antigens represented by peaks II, IV, VI and VII of RBL cell surface components by intact RMC (Fig. 28b) may indicate the presence of common as well as specific antigens in the same regions of the gel. Peak I_{RMC} reacted with anti-RBL_{abs} (Fig. 20a) but was unaffected by absorption of anti-RMC with intact RBL cells. Therefore, it most likely represents a common antigen which is not present on the outer surface of the plasma membrane of RMC or RBL cells. Based on all the available data, the most obvious specific surface antigens of RMC and RBL cells are those appearing in peaks V_{RMC} and III_{RBL}, respectively (Fig. 28). Both peaks were unaffected by the absorption in terms of profile and intensity. Furthermore, V_{RMC} did not react with anti-RBL_{abs} and neither did III_{RBL} with anti-RMC_{abs} (Fig. 20).

GENERAL DISCUSSION

The present study has clearly demonstrated that antibodies to RMC cross-reacted with RBL cells and vice versa. After appropriate absorption, anti-RMC_{abs} and anti-RBL_{abs} were still cytotoxic to both cell types (Fig. 6). Binding between the antibodies and the two cell surfaces was first demonstrated by indirect immunofluorescent staining (Table IV). The abilities of both antisera to induce an immediate hypersensitivity type of skin reaction (Table V) and to block IgE binding to both cell types (Fig. 10) indicated that the cross-reacting antibodies might be directed against the receptors for IgE. While the present study was in progress, antibodies to the receptors for IgE had been induced in several other laboratories. Studies on both anti-RMC_{abs} and anti-RBL_{abs} are different from those other antisera because both sera had been shown to contain antibodies to the individually unique antigens as well as common antigens, in addition to those specific for the receptors for IgE. The presence of antibodies to RMC or RBL cell specific antigens in either serum was indicated by the findings that each of the two antisera reacted more strongly to its 'homologous' cell type than the 'heterologous' counterpart. There was only one antiserum which could be regarded as closely similar to the anti-RBL serum of this study. The antiserum produced by Isersky et al. (1977) was raised in a rabbit which was inoculated with whole RBL cells emulsified with complete Freund's adjuvant. As mentioned before, this antiserum could inhibit IgE binding to RBL cells and rat peritoneal mast cells. Since

it was capable of precipitating free receptors as well as IgE-receptor complexes, the antiserum was believed to contain antibodies to two distinct determinants of the receptor for IgE. However, no attempt was made to ascertain whether the antiserum contained any antibodies to either the RBL cell specific antigens or the other common antigens possibly shared by both RMC and RBL cells. All the anti-receptor sera described so far were induced in rabbits injected with either IgE-receptor complexes (Ishizaka et al., 1977b) or partially purified receptor preparations (Isersky et al., 1978; Conrad et al., 1979). While Ishizaka's antiserum precipitated mainly a receptor component of 45,000 daltons (Conrad et al., 1978), Isersky's antiserum was found to react with a receptor molecule of 58,000 daltons, and Conrad's antiserum reacted more strongly to the 55,000 dalton component than the 45,000 dalton receptor. However, studies on antibodies against surface antigens other than the receptor for IgE of either rat mast cells or rat basophilic leukemia cells were not elaborated in these investigation. Both antisera of the present study were shown to contain antibodies to the 55,000 and 45,000 dalton receptors for IgE (Figs. 24, 26 & 27). In addition, they precipitated several other surface molecules from RMC and RBL cells. Although the 58,000 dalton component of the receptor recognized by Isersky's antiserum was derived from a different RBL cell line, recent collaboration between the two laboratories revealed that it has the same properties as R (45,000 daltons) but a molecular weight similar to H (55,000 daltons) (Froese, personal communication). Both the 45,000 dalton (R) and 55,000 dalton (H) receptors were obtained from the cell line maintained in Dr. A. Froese's laboratory. Therefore, both antisera of the present study clearly reacted with the same receptor

molecules (H and R) as those recognized by Conrad's antiserum. In addition, the serum prepared by Ishizaka et al. (1977b) was also shown to react with the R receptor (Conrad et al., 1978).

During the course of the present study, the author had the chance of analyzing both Drs. Ishizaka's and Conrad's anti-receptor sera (designated as anti-RBL_p and RAR, respectively) together with anti-RMC_{abs} and anti-RBL_{abs}, and the results were compared. One of the results came from the indirect immunofluorescence study. It was found that under the same experimental condition, RAR did not stain either RMC or RBL cells, while anti-RBL_p stained both with strong fluorescent intensity. The results for anti-RMC_{abs} and anti-RBL_{abs} were similar to the ones shown in Table IV. When mixed peritoneal cells were used as targets, anti-RBL_p clearly stained only RMC and not the other cell types found in the peritoneal exudate. In another study, RAR weakly elicited a skin reaction as described in Table V in normal rats while anti-RBL_p always turned out positive results even at very diluted concentrations, e.g., 1 : 200. The antibody titre of anti-RBL_p capable of inducing the skin reaction was similar to those of anti-RMC_{abs} and anti-RBL_{abs}. However, the skin blueing reaction induced by the former in the presence of Evan's blue appeared almost immediately whereas that induced by the latter two took at least eight minutes to develop. The above data clearly demonstrate that anti-RBL_p was a stronger antiserum than either anti-RMC_{abs} or anti-RBL_{abs}. The potent activity of the former serum was partly due to the fact that it had been purified by adsorption onto and elution from RBL cells. A comparison between the potency of RAR and an anti-RBL serum has been reported in the study by Conrad et al. (1979). It should be noted that the anti-RBL serum in

that study was in fact the same one used for the present project. It was found that anti-RBL_{abs} was more cytotoxic and more inhibitory on IgE binding to RBL cells than RAR. On the other hand, both RAR and anti-RBL_{abs} were capable of precipitating IgE-receptor complexes indicating that they both contained antibodies to determinants distant from the IgE binding site of the receptor component. There is evidence that RAR contained more antibodies to the R component than anti-RBL_{abs}. Under similar experimental conditions including the amounts of materials used for the analysis, RAR precipitated roughly 65% of the complexes whereas anti-RBL_{abs} only bound 17%. This was to be expected since RAR was induced in a rabbit injected with partially purified receptor preparations while the antigens used for producing anti-RBL_{abs} consisted of whole RBL cells.

Analysis of the surface antigens bound by either anti-RMC_{abs} or anti-RBL_{abs} revealed that individual cell specific antigens were present together with the common surface antigens shared by both RMC and RBL cells. Hence, anti-RMC_{abs} reacted with at least eight RMC components (Fig. 11a) and mainly two RBL cell surface antigens (Fig. 20b). On the other hand, anti-RBL_{abs} precipitated at least seven RBL cell surface antigens (Fig. 13a) and about four RMC surface antigens (Fig. 20a). Two of the antigens precipitated by both antisera had been clearly identified to be the 55,000 and 45,000 dalton components of the receptors for IgE. The different amounts of common surface antigens (as measured by cpm) bound by either antiserum indicated that not all antigenic molecules were equally expressed on the surface of either cell. In addition, all the surface molecules were not equally immunogenic. For instance, the 45,000 dalton receptor for IgE is more domin-

ant than the 55,000 dalton receptor on the RMC surface (Froese, 1979). However, when both components were isolated from the rest of the surface molecules by means of IgE-affinity chromatography, anti-RMC_{abs} was found to react more with the 55,000 dalton component than the 45,000 dalton molecule (Fig. 24d). The finding suggested that although the former component was present in fewer numbers, it was more immunogenic than the latter one. In another instance, anti-RBL_{abs} precipitated a 130,000 dalton component from the RMC surface antigens (Fig. 20a) but anti-RMC_{abs} did not seem to bind any RBL cell surface molecule of similar m.w. (Fig. 20b). This observation indicated that while the component was, no doubt, present on both cell surfaces, anti-RBL_{abs} seemed to contain more antibodies directed against it than anti-RMC_{abs}. It would imply that the immunogenicity of the corresponding determinant on this molecule on the RMC surface was different from that of the RBL cell surface. Absorption studies using either RMC or RBL cells to remove antibodies to the common antigens, revealed that there were at least six common antigens shared by both cell types (Figs. 28a & 28b) spanning a m. w. range from 30,000 to 175,000 daltons. In a separate study, the crude anti-RBL serum produced by Ishizaka et al. (1977b) was shown to precipitate three extra antigens from RBL cells of the same culture line used in the present study (Conrad et al., 1978). The experimental conditions were also similar to the ones used in this study. It was found that surface molecules precipitated by the crude anti-RBL serum seemed to appear in fractions where certain antigens bound by anti-RBL_{abs} did appear, i.e., peaks I, III and VI of Fig. 13a. It is not known whether the similarity in electrophorectic mobility of the molecules precipitated by two different sera is just a coincidence or

it actually reflects the presence of identical molecules. These antigens described by Conrad et al. (1978) may have been associated with the IgE-receptor complexes with which the animal was injected. This could, in turn, suggest that they may be in close contact with the receptors. Hence, during the solubilization with NP-40, small amounts (enough to be immunogenic) remained in contact with the receptors and were isolated with them. Antibodies to these antigens were partially removable from the crude anti-RBL serum by absorption with rat peritoneal exudate cells that had been depleted of RMC (Ishizaka et al. 1977b; Conrad et al., 1978). Hence, it would appear that some of the antigens were shared by RBL cells, peritoneal cells and possibly RMC. On the other hand, both peaks I and III of the RBL cell surface antigens were still precipitated by anti-RBL_{mas} (Fig. 28b). The results seemed to indicate that the antigenic determinants of both components may be buried in the plasma membrane or that they are RBL cell-specific antigens. However, since peak III_{RMC} had been shown to have a m.w. (130,000 daltons) similar to peak I_{RBL} and the former was also precipitated by anti-RBL_{abs} (Fig. 20a), it is most likely that both peaks represent the same antigen with hidden determinants. Peak III_{RBL} (78,000 daltons) which did not react with anti-RMC_{abs} (Fig. 20b) most likely represents one of the RBL cell-specific antigens. As for anti-RMC_{abs}, it is rather unfortunate that there is no other anti-RMC serum of similar nature with which it can be compared. Most of the anti-RMC sera reported were not used to study either the RMC surface antigens or the receptors for IgE (Chapter I, Section VI). The anti-RMC_{bas} serum of this study had characterized the RMC-specific antigen(s) to be (a) component(s) with an apparent m.w. of 85,000 daltons (peak V_{RMC} in Fig.

28a).

As has been mentioned in Chapter I, the rat cell surface contained large numbers of histocompatibility antigens and differentiation antigens. Lymphocyte differentiation antigens were shown to be abundant in the membrane of rat peritoneal exudate cells whereas they were rarely found on the surface of liver cells or erythrocytes (Williams and Standring, 1977). Hence, absorption of the antisera with rat liver cells would only remove antibodies to the rat histocompatibility antigens. However, it is possible that the absorption may have left behind small traces of these particular antibodies, enough to precipitate the abundant histocompatibility antigens that were present in the detergent extract of RMC or RBL cells. Hence, it should not be surprising to find the presence of these antigens among the precipitated, common surface antigens shared by both RMC and RBL cells. The molecular weights of most of the rat histocompatibility antigens were estimated to be around 30,000 to 40,000 daltons (Callahan and DeWitt, 1975a & 1975b; Williams and DeWitt, 1976). However, neither anti-RMC_{abs} nor anti-RBL_{abs} precipitated any surface antigens of such molecular weights from rat lymph node cells which usually contain a lot of histocompatibility antigens on their surfaces (Figs. 12b, 12c & 14b). Therefore, antibodies to rat histocompatibility antigens are possibly not present in either antiserum of the present study. Most rabbit anti-rat lymphoid cell sera seemed to contain a large amount of antibodies specific for lymphocyte differentiation antigens which included Thy-1 antigens, T lymphocyte-specific antigens and leucocyte common antigens (Williams and Standring, 1977). Hence, it is possible that the anti-RMC and anti-RBL cell sera of this study may also contain antibodies

against some of the common differentiation antigens shared by both RMC and RBL cells. However, it is difficult to determine, within the scope of the present study, which ones of the already known common surface antigens of both cell types are differentiation antigens. In view of the fact that most of the rat lymphocyte common differentiation antigens have molecular weights ranging from 130,000 to 200,000 daltons (Standring et al., 1978), it is possible that both peaks III_{RMC} and I_{RBL} (m.w. of 130,000 daltons) may represent the common differentiation antigens of RMC and RBL cells. The presence of specific differentiation antigens in the membrane of a particular cell type has long been studied. For instance, Thy-1 antigens (m.w. of 25,000 - 27,000 daltons) are specific markers for thymocytes whereas the T lymphocyte-specific antigens (m.w. between 70,000 and 100,000 daltons) have been found solely on the surfaces of peripheral T cells and thymocytes (Bustin et al., 1972; Ladoulis et al., 1974; Ishii et al., 1976; Williams and Standring, 1977). The presence of rat liver cell specific antigens had also been demonstrated (Behrens and Paronetto, 1977). Hence, it is plausible to suggest that the two non-cross-reacting surface antigens represented by peaks III_{RBL} (m.w. of 78,000 daltons) and V_{RMC} (m.w. of 85,000 daltons) could most likely be the specific antigens of RBL cells and RMC, respectively.

The presence of surface immunoglobulins has been identified in the membrane of rat lymphoid cells (Ladoulis et al., 1974; Williams and DeWitt, 1976). The molecular weights of rabbit IgG and rat IgG have been estimated to be around 169,000 (Sober and Harte, 1973) and 156,000 (Bazin et al., 1974) daltons, respectively. Since neither anti-RMC_{abs} nor anti-RBL_{abs} precipitated rat IgE (Table III), it is unlikely

that any surface immunoglobulins would be present among the precipitated surface antigens of both cell types. It is even unlikely that the rabbit antibodies, most of which belonged to the IgG class, could be radioiodinated just by binding to the radio-labelled cell surface antigens. Nevertheless, the peaks which one would suspect to represent immunoglobulins would be peaks I_{RMC} and II_{RMC} with m.w. of 175,000 and 160,000 daltons, respectively. However, reduction of the precipitated RMC surface antigens did not change the overall profile of the peaks. Therefore, it can be ruled out that these two peaks represent any immunoglobulins.

The possibility of inducing anti-rat Ia sera (Soulillou et al., 1976; Mossmann et al., 1979) suggests the presence of Ia antigens in the membrane of rat lymphoid cells. Such antigens of the rat have recently been characterized. McMaster and Williams (1979) reported that the rat Ia glycoprotein complex was composed of two non-covalently linked polypeptide chains of apparent m.w. 30,000 and 24,000 daltons as determined by SDS-PAGE analysis. However, there were some indications that mast cells lacked Ia antigens (Mossmann et al., 1979; Daëron and Voison, 1979). Anti-rat β_2-microglobulin sera had been produced (Isersky et al., 1977; Mossmann et al., 1979) but the antigens have not yet been fully characterized in the rat. Studies on murine β_2-microglobulins revealed that they have a molecular weight of 12,000 daltons (Vitetta and Uhr, 1975). Analysis of the precipitated surface antigens of RMC or RBL cells by SDS-PAGE did not reveal any significant amount of light molecular weight components. Hence, it is not certain if rat Ia antigens and β_2-microglobulins are present among the precipitated surface antigens of RMC and RBL cells.

There is no evidence that lactoperoxidase (m.w. of 77,500 daltons) which was used for catalyzing the reaction of surface labelling could be self-labelled. Since the labelled cells had always been washed thoroughly to eliminate any trace of free radio-labels before detergent extraction, it is highly unlikely that lactoperoxidase could be present among the precipitated surface antigens of this study. The 78,000 dalton component precipitated by anti-RBL_{mas} is probably a unique antigen of the RBL surface. However, it is not certain whether it belongs to a class of specific differentiation antigens or a group of tumor-specific antigens. It is not unusual for chemically induced tumor cells to possess distinctly tumor-specific antigens on their cell surfaces. Hence, it is possible that some of the antibodies of anti-RBL_{abs} were directed against these antigens. Since there is no anti-rat basophil serum to compare with, it is difficult to demonstrate the presence of tumor-specific antigens on the surface of RBL cells.

It should be mentioned that although both anti-RMC_{abs} and anti-RBL_{abs} contained anti-receptor antibodies, the numbers were too small to be able to mimic the activity of IgE. In other words, neither serum could induce a non-cytotoxic histamine release from target cells. This is based on the author's undocumented observation that both antisera required the presence of complements in order to induce degranulation of RMC. As has been mentioned in Chapter I, there was only one anti-receptor serum which was capable of inducing a non-cytotoxic histamine release from RMC in vitro (Ishizaka et al., 1977b). This antiserum had been shown to contain a large amount of antibodies directed against determinants that were close to the IgE-binding site of the receptor molecule. However, both antisera of the present study could be useful

for other studies. For instance, they could be used for suppressing the mediator release from the target cells. They could block IgE binding to these cells without drastically altering the cellular contents in vitro. The $F(ab')_2$ fragments of the antibodies would do the same thing in vivo by avoiding the cause of cell lysis even in the presence of complement which mainly bind to the Fc portion of the antibody molecule (Dorrington and Painter, 1974). On the other hand, both antisera can be used for studying the origins of RMC and basophils. There was indication that both cells originated from different anatomical compartments but their exact origins are still debatable. Since both antisera have the potential to recognize specific as well as common antigens of RMC and RBL cells, they can be employed to detect the presence of mast cells or basophils in different anatomical compartments at the very early beginning of the animal life. The differentiation antigens of either cell type have not yet been clearly identified. However, the specific antigens, characterized by the two antisera of the present study, could be used as markers for the presence of their corresponding cell types. The statement is based on the assumption that the 78,000 dalton component of RBL cells was a specific differentiation antigen. On the other hand, RBL cells are neoplastic cells and hence, like most of them, they may contain modified surface antigens. It would be interesting to find out which of the surface molecules are affected by the modification and how the changes are related to the mechanisms or biochemical pathway of mediator release of RBL cells. Normal basophils could serve as a control for the study. Again, the antisera would prove to be useful for the purpose.

SUMMARY

Anti-rat mast cell (RMC) and anti-rat basophilic leukemia (RBL) cell sera were induced in rabbits by injecting them with the appropriate cells in Freund's complete adjuvant. All antisera were heated at 56°C for 30 minutes and were absorbed five times with rat liver cells. The anti-RMC sera were further absorbed with IgE-Sepharose. Three anti-RMC sera were induced. The one that was most reactive to RMC surface antigens in terms of cytotoxicity (Fig. 1) as well as in its ability to inhibit IgE binding (Fig. 2) and to precipitate a surface component of similar apparent molecular weight (m.w.) as that of the receptor for IgE (Fig. 3) was selected for subsequent studies. Based on its cytotoxic activity (Fig. 4) and reactivity to RBL cell surface antigens (Table II and Fig. 5), one of the three anti-RBL cell sera produced was selected for further studies. Both selected antisera, anti-RMC_{abs} and anti-RBL_{abs}, were not cytotoxic to rat lymph node cells (Fig. 6) but still killed both RMC and RBL cells (Fig. 7) in the presence of complement. Binding between antibodies present in these antisera and the surfaces of both cell types was demonstrated by indirect immunofluorescence (Table IV and Fig. 8). The above results suggest that both antisera cross-reacted with RMC and RBL cells. The nature of the cross-reacting antibodies was revealed by the finding that they could induce skin reactions in normal rats (Table V and Fig. 9) as well as inhibit IgE binding to both cell types (Fig. 10). Since neither a normal rabbit serum nor a rabbit anti-rat sarcoma cell serum absorbed with rat liver cells had similar effects

as those above, these activities were deemed to be specific. These data suggested that both antisera might contain antibodies against the receptors for IgE. In general, each of the two antisera was found to be more reactive to its 'homologous' cell type, e.g., anti-RMC_{abs} versus RMC, than to its 'heterologous' counterpart, e.g., anti-RMC_{abs} versus RBL cells.

Surface antigens of RMC or RBL cells were radioiodinated followed by solubilization with Nonidet P-40. They were then reacted with the appropriate antiserum and the immune complexes were insolubilized with protein A-Sepharose followed by analysis with SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The results showed that while anti-RMC_{abs} precipitated at least eight RMC surface antigens (Fig. 11a) and mainly two RBL cell surface components (Fig. 20b), anti-RBL_{abs} reacted with at least seven RBL cell surface antigens (Fig. 13a) and about four RMC surface components (Fig. 20a). Most of these antigens were found only on the surface of RMC or RBL cells but not on rat lymph node cells (Figs. 12 & 14). Furthermore, fetal calf serum which was present in the culture medium for RBL cells was shown to have no effect on the reaction between anti-RBL_{abs} and RBL cells (Figs. 15 & 16). Both antisera were shown to react with RMC or RBL surface antigens among which were the receptors for IgE. They both precipitated the 55,000 and 45,000 dalton receptors of RBL cells which had been isolated through adsorption to and elution from IgE-Sepharose (Fig. 24). Since they could precipitate free receptors (m.w. 45,000 daltons) (Fig. 24) as well as IgE-receptor complexes (Figs. 26 & 27), it was concluded that some of the cross-reacting antibodies were directed against at least two different antigenic determinants on the receptor molecules,

one close to the IgE binding site and one distant from it.

Additional studies were performed with the antisera after absorption with the cross-reacting cell type (e.g., absorbing the anti-RBL serum with RMC). In addition to the antibodies against the receptors for IgE, both antisera were shown to contain antibodies to at least four other surface antigens shared by both RMC and RBL cells. Their molecular weights were found to be in the range of 30,000 to 175,000 daltons. The different amount of common antigens (as measured by cpm) bound by either antiserum indicated that not all antigens were equally immunogenic or equally expressed on the surfaces of RMC and RBL cells. The absorption studies also revealed that the anti-RMC serum used in this study reacted with one specific surface antigen of RMC (m.w. 85,000 daltons) (Fig. 28a). By the same means, the anti-RBL cell serum was shown to contain antibodies against one RBL cell-specific antigen (m.w. 78,000 daltons) (Fig. 28b). Hence, it was concluded that both anti-RMC_{abs} and anti-RBL_{abs} contained antibodies to RMC and RBL cell specific antigens, respectively, as well as antibodies against common surface antigens, including the receptors for IgE, shared by the two cell types.

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