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CHARACTERIZATION OF ANTISERA TO RAT PERITONEAL MAST CELLS

BY

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Preface

The main aim of this study was to obtain a rabbit antiserum specific for rat peritoneal mast cells and to establish whether or not such a serum contained antibodies to the receptor for IgE. The availability of antibodies to this receptor may prove useful in the characterization of the receptor itself.

This thesis is divided into three chapters. The first two chapters are introductory in nature and contain a literature survey. The third chapter covers the experimental work performed by the author.

The first chapter deals with the general aspects of mast cells. The role of mast cells in immediate hypersensitivity is introduced in the last part of this chapter, where the phenomenon of degranulation and mechanisms of histamine release are discussed.

The second chapter concentrates on studies on the surface of mast cells. It covers the literature on the binding between mast cells and IgE molecules as well as the characterization of receptors for IgE. The last part of this chapter is a general review of previous studies on anti-rat mast cell sera.

The final chapter is divided into two parts. The first section contains the methodolody of the author's work. Results of this work are presented and discussed in the last part of chapter III.

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CHAPTER I

THE MAST CELL

Introduction

The study of mast cells as unique entities is almost one hundred years old. It was P. Ehrlich (1879) who first described a group of cells exhibiting a strong basophilic metachromatic behaviour. These cells were packed with granules. They were recognized in certain connective tissues and were distinct from the larger mononuclear cells. He called these cells 'Mastzellen' because they seemed most numerous in areas of increased local nutrition, e.g. during chronic inflammatory states. He later found that there were similar cells present in the peripheral blood. These blood basophils seemed to derive from the bone marrow, in contrast to the previously described 'Mastzellen' which were present only in connective tissues.

Since Ehrlich's discovery, morphological studies of mast cells and basophils have accumulated owing to improvements in microscopy as well as in histological techniques. Mast cells or blood basophils are characterized by the basic metachromatic staining reaction of their granules. For instance, a bluish dye like toluidine blue stains the granules of mast cells purplish red while thionine, another bluish dye, stains them reddish violet. Dyes that have been employed to stain mast cells metachromatically are e.g. thionine, toluidine blue, methylene blue, neutral red, safranine and azure. Of these, the blue dyes give a reddish, the red dyes a yellowish tone to the granules. Recently two new staining methods have been used to stain human tissue mast cells. Callerane and Condemi (1974) used the dye alkaline Giemsa while Hodinka

and Csaba (174) used the Black-Ansley silver staining techniques. Both groups of workers claimed to be able to demonstrate tissue mast cells distinct from other cells.

Morphology of Mast Cells

Techniques in electron microscopy and histochemistry have indeed contributed a lot to the understanding of the morphology of mast cells. Mast cells generally have an ovoid or elongated shape. In rats, for instance, they are seen either in an oval or polymorphous form. Benditt (1958) reported that polymorphous mast cells were found adjacent to blood vessels and in the connective tissues. Mast cells from the mesentery appear oval while those from the peritoneal cavity are mainly spherical. The nucleus of mast cells has a double-layered membrane and is usually round or kidney-shaped. One or more nucleoli may be present. The intranuclear substances seem to be homogeneous in nature with aggregated chromatin. The bulk of the cytoplasm is filled with the characteristic membrane-bound 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 of mast cells. The mast cell membrane is thin and is characterized by the presence of small villous processes.

Size and Distribution of Mast Cells

The size of mast cells varies among different species. Mast cells are large and numerous in the capsule of ox liver (Holmgren and Wilander 1937) but small and scattered in the liver of dogs (Nagayo 1928) while the livers in rabbits and rats are almost devoid of mast cells (Westphal 1891). Rat mast cells in suspension have a mean diameter of

12.6 μ and a round nucleus of 5 μ in diameter.

Westphal (1891) studied the distribution of mast cells in certain species and found that they were numerous in cat, dog, goat, rat, bat and calf but rare in rabbit and guinea pig. Later Zimmerman (1908) using improved histological techniques showed that tissue mast cells are also common in guinea pig. Generally, mast cells are found in the loose connective tissue and around blood vessels, in the interstitium of the myocardium, between fat cells, in the peritoneum, in most organ capsules and in the thymus.

Ontogeny of Mast Cells

Studies of the development and maturation of mast cells have been performed mainly in the rat. Maximow (1910) and Alfejew (1924) showed that tissue mast cells are first recognizable in the early embryo of the rat as small lymphoid-like cells with few granules. They arise either from fixed mesenchymal precursors or from basophilic non-granular cells which settle down and undergo differentiation in the loose subcutaneous and intramuscular connective tissues of the head and the neck. The number of mast cells increases, reaches a maximum, in the two days just before birth. In the new-born rat the distributuion of the mast cell population is similar to that in the mature rat, but the number of mast cells per unit volume of connective tissue is much higher. Mast cells are rare in the parenchymatous organs of the rat in contrast to their great abundance in connective tissues and the peritoneum. There are virtuallly no mast cells present in spleen, kidney and within the brain substance of the rat.

The mast cell precursors are indistinguishable from other primitive mesenchymal cells. Combs (1966) described a four-stage maturation for

tissue mast cells based on morphological and histochemical data. The first two stages are capable of mitotic proliferation and the last two are not. The first detectable materials that contribute to the formation of the granules are intimately associated with the Golgi Apparatus, in the form of small and dense granules inside smooth, membrane-limited vesicles. These 'progranules' appear to aggregate and migrate to the more peripheral parts of the cytoplasm. The mast cell synthesizes a finely granular material which aggregates with the 'progranules'. The whole complex increases its size by aggregation until it reaches a size of 0.5 - 1 μ in diameter. The formed dense and chemically homogeneous granule is wrapped within a perigranular membrane.

The Mast Cell Granules

The mast cell is packed tightly with granules. Each cell, on the average, contains some 500 granules (Uvnäs 1964). The number and size of granules varies among different species. They are comparatively coarse in rat, mouse and guinea pig, while small in birds and amphibia (Westphal 1891). They have a round, oval or irregular form as seen under the electron microscope. In some species, the granules appear to be homogeneous electron-microscopically while in others a lamellar structure has been observed.

Chemical Contents of the Granules

The era of biochemical studies of mast cell did not appear until the 1930's. Lison (1935) suggested that the metachromasia displayed by mast cells in the presence of a basic dye, e.g. toluidine blue, was indicative of the presence of compounds containing ester sulfate radicals. An anticoagulant named heparin was extracted by McLean from the dog liver in 1916. Holmgren and Wilander (1937) showed that heparin was present in organs where mast cells were abundant. Cartilage, the

only other common tissue component which stains metachromatically is almost devoid of anticoagulant activity. Jorpes et al. (1937) connected the presence of considerable amounts of sulfate in heparin with the metachromasia of mast cells. Together with previous findings, the data suggest that mast cells contain heparin. Since it is the granules of mast cells that stain metachromatically, it was put forward that heparin is located within the granules. Hence, heparin has been extracted, analysed and quantitated from mast cells by various workers. Histochemical data show that heparin is an acid mucopolysaccharide with a molecular weight of 22,000 daltons and it is present the granule together with a network of basic protein. In rat mast cells, heparin accounts for 2.7 - 4.6% of the cellular volume (Benditt, 1957) or around 25% of dry weight of the cell (Schiller, 1963). Young mast cells contain only poorly sulfated mucopolysaccharides. Sulfation increases in parallel with maturation. Normal rat mast cells are capable of incorporating sulfate compounds but exhibit a low turnover rate.

Anaphylactic shock is a well established phenomenon in animals receiving an intravenous injection of a foreign protein. It was shown that histamine is the main mediator associated with the reaction. In dog, both heparin and histamine are released from the liver which is rich in tissue mast cells. During the release of both substances, tissue mast cells in liver lose their metachromatic material (Wilander 1938). A series of studies performed by Riley et al. (1952) have indicated the existence of a consistent correlation between histamine content and mast cell number in a variety of tissues. It has been observed, moreover, that the phenomenon of mast cell degranulation is often followed by histamine release. Chemicals which cause a local damage of mast cells

produce a complete depletion of histamine locally. Further application of a histamine liberator has no effect on the cells. Studies on isolated mast cell granules reveal that histamine is stored in the granules together with heparin. In rat mast cells, histamine accounts for 1 - 2% of the cellular volume (Benditt 1958) or 9 - 10% of the dry weight (Benditt 1964).

Serotonin, or 5-hydroxytryptamine (5-HT), was first identified in rat mast cells by Benditt et al. in 1955. It is released together with histamine and accounts for 0.06% of the total mast cell volume (Benditt 1958). Mouse mast cells also contain 5-HT while normal mast cells of other species do not.

In 1940, Kellaway and Trethewie described the release during the antigen-antibody reaction in sensitized tissue mast cell of a substance causing slow contraction of the smooth muscle. The substance is called the slow reacting substance or SRS. It has been proposed that SRS is a product split off from the mast cell membrane by enzymatic attack (Uvnäs et al. 1960).

Recent evidence shows that mast cells of human lung tissue release an eosinophil chemotactic factor (ECF) which accumulates esinophils at the site of an allergic reaction. Other chemicals such as hyaluronic acid and dopamine (3-hydroxytryptamine) have been reported to be present in mast cells of some species (Asboe-Hansen, 1950; Falck et al., 1959).

Enzymes within the Mast Cell

Besides chemical mediators, mast cells have been shown to contain numerous enzyme systems, particularly those that are involved in the formation of granules and their chemical mediators. For instance, histidine decarboxylase is present in mast cells. Histamine is formed

locally by the catalytic reaction of the above system. The presence of 5-hydroxytryptophane decarboxylase has been demonstrated in the mast cells. Phosphatidase A has been shown to be active during mast cell degranulation. This hydrolytic enzyme splits off unsaturated fatty acids from phospholipids. Uvnäs et al. (1963) suggested that the activity of this enzyme is normally suppressed in mast cells. When the inhibitory factor is removed the activated enzyme may trigger a process which finally results in degranulation. It was thought that histamine liberators like compound 48/80 might exert their effect by removing an enzyme inhibitor and thus activating such a lytic enzyme. An esterolytic, chymotrypsin-like peptidase is also found in rat mast cells. Lagunoff and Benditt (1963) proposed that this enzyme may act on the lipoprotein of the mast cell membrane, and hence functions as a trigger mechanism in the process of degranulation. Apart from the previously mentioned enzymes, adenyl cyclase, ATPase, acid phosphatase and leucine aminopeptidase are also reported to be active in mast cells.

Functions of Mast Cells

The release of heparin from mast cells may disturb blood coagulation but this occurs only in rare pathological states. Mast cells might influence lipid turnover of the tissue since lipid transport is accelerated by a small amount of heparin. Engelberg's studies (1961) in humans have indicated that a relative deficiency of circulating heparin may lead to a raised level of serum lipid. Fodor et al. (1962) showed that there was an increased secretory activity of mast cells in animals after the administration of high fat diets. Heparin alters the activity of the cells of the connective tissue, the synthesis of collagen and it inhibits the enzyme hyaluronidase. Riley et al. (1963) proposed that mast cells are important in maintaining the structural integrity of

connective tissue. Following injury or inflammation of the connective tissues, mast cells disrupt and release their granular content. The local fibroblasts and macrophages are activated to digest heparin and heparin containing granules. The undigested heparin is converted into mucopolysaccharide ground substance, from which extra-cellular collagen fibrils may be formed. Hyaluronic acid possesses strong water binding properties and thus may largely determine the permeability of the connective tissue. The edema associated with injury to connective tissue causes mast cell degranulation and the released hyaluronic acid will then bind the edema fluid and change it into a mucinous gel. The normal accumulation of mast cells around small blood vessels is consistent with the view that the function of mast cells is linked to the vascular permeability. Based on this, Asboe-Hansen and Wegelius (1957) proposed that one of the functions of mast cell is to change a perivascular edema into mucinous ground substance.

Histamine is readily released by the mast cell. This may lead to a vasomotor reaction which causes vasodilation and increases the capillary permeability. The secretion of the proteases from mast cells may serve another important function. Histamine may perhaps catalyse the synthesis of biologically active kinins by these proteases.

Mast cells may participate in body defense mechanisms. The number of human mast cells has been shown to increase in acute inflammatory diseases or in the acute phase of chronic inflammatory conditions, e.g. Rheumatoid Arthritis (Boseila et al. 1961). In a study of the rapid initiation of acute inflammation at a site of injury in rats, Sheldon and Bauer (1960) found that degranulation of the mast cells prior to experimental infection somewhat delayed the inflammatory response

and slightly diminished the host's resistance.

Mast cells also participate in reactions to parasitic invasions. Coleman and De Salva (1963) examined the cyst wall of the larval cestode parasite (Hydatigena Taenial Formis) in experimentally infected rats. The outer two layers of the cyst wall contained mast cells and the number of mast cells in the liver as a whole was greatly increased. They thought that the entire cyst wall was of host-inflammatory origin and mast cells were in someway helping the body-defence mechanism. Hogarth-Scott and Bingley (1971) showed that damage to mast cells prolonged the infection of rats with the nematode Nippostrongylus Brasiliensis.

Mast Cells and Immediate Hypersensitivity

Mast cells take part in immediate hypersensitivity by degranulating with the subsequent release of the chemical contents of the granules. Histamine, heparin, SRS-A and ECF are mainly released. The biological consequence caused by the release of these substances have been mentioned previously. For almost 15 years, the morphological changes of mast cells during degranulation caused either by artificial histamine liberators, e.g. compound 48/80, or the interaction of antigen/allergen with cell-bound IgE have been carefully traced using elaborate histochemical techniques. Electron microscopy is one of the main tools for such studies. Accurate spectrofluorometric measurements have made it possible to estimate the amount of histamine released from sensitized mast cells. Owing to the availability and simplicity of obtaining a relatively high number of mast cells from the peritoneum of the rat, many studies have been performed on the mast cells of this species. However, in man, blood basophils, which can be enriched considerably, have been used mainly for studies on the release of mediators.

Mast cells isolated from immunized rats will degranulate and release their chemical mediators when challenged with the specific antigen in vitro. Hisfamine is also released from basophils of atopic individuals when they are incubated with the specific allergen (Lichtenstein and Osler, 1964). It is possible to sensitize normal rat mast cells or normal human basophils passively by incubating the cells with reaginic antibodies, e.g. IgE, obtained from immunized rats or atopic individuals, respectively. The sensitized cells are then challenged with the specific antigen/allergen and histamine is released from the target cells. Morphologically, the challenge leads to cell degranulation. Ishizaka et al. (1971) have shown that anti-IgE antibodies also can induce histamine release from sensitized basophils from atopic or normal humans.

Skin mast cells also serve as target cells for IgE. In atopic individuals, an intracutaneous injection of the specific allergen will cause the almost immediate formation of wheal and erythema in the skin site. This does not happen in the case of individuals who are not allergic to a particular allergen. The skin of a normal individual can be sensitized to a certain allergen by injection of reaginic antibodies obtained from an atopic subject. When the skin site is then challenged with the specific allergen, the wheal and erythema reactions will develop in the normal individual. Reactions of this kind are known as the Prausnitz-Küstner (P-K) reaction (Prausnitz and Küstner, 1921). Animal models are used in studying the passively sensitized mast cells in vivo. Mouse, rat, dog and guinea pig are the most common choices. The basic principle is the same as mentioned above. Histamine release from the sensitized mast cells leads to increased vascular

permeability which is made visible by a dye (Evan's Blue) which is injected along with the antigen intravenously into the challenged animal. This reaction is commonly known as the passive cutaneous anaphylaxis (PCA) reaction (Ovary, 1958).

Mast Cell Degranulation

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). The process requires energy (Uvnäs, 1967). This seems to lead to the formation of 'pores' within the cell membrane and it is presumably as a result of a widening of these pores that the granules now, lacking their membrane, move freely to the exterior of the cell. A few seconds after the exposure of mast cells to the histamine liberator, granules are exteriorized by the cells. Most of the extruded granules adhere to their 'mother' cell thereby concealing the cell membrane. The normally round and smooth cell loses its transparency and becomes a non-transparent 'mulberry'-like body. Only a few granules appear in the suspension medium (Uvnäs, 1967).

Hastie (1971) reported that the morphological change during degranulation of human basophils from an atopic patient on challenge with the specific allergen are observed to be fundamentally comparable to those induced in rat mast cells by compound 48/80.

Uvnäs (1973) tried to correlate morphological and biochemical events of antigen-induced histamine release from rat mast cells. 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 extracellular fluid. Uvnäs (1973) demonstrated that there was a correlation between the time that histamine is released and the time when mast cells degranulate.

Mechanisms of Histamine Release

The mechanism of antigen-induced histamine release from mast cells or basophils is still somewhat controversial. There are many hypotheses for the mechanism; only the generally accepted ones will be mentioned here. Histamine release from sensitized target 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 lysis. It requires metabolic energy since it was shown that metabolic inhibitors like those for the glycolytic pathway inhibit histamine release from the target cells. Histamine release is also dependent on the presence of Ca^{++} and Mg^{++} ions in the reacting system.

The sequence of events leading to the release of histamine begins with the firm binding of the sensitizing antibody to the target cell through the receptor on the cell surface. Ishizaka et al. (1970a) showed that only the Fc fragment of human IgE, but not the $F(ab')_2$ fragment, could sensitize primate lung tissue passively and the actual binding of the Fc fragment to the human basophil was demonstrated by using radio-labelling techniques (Ishizaka et al., 1970b). Bifunctional or polyfunctional antigenic/allergenic molecules can induce histamine release from the sensitized target cells while unifunctional molecules

cannot. Even divalent haptens can trigger sensitized mast cells (Mossmann et al., 1974). The bridging of adjacent cell-bound IgE molecules by specific allergen is thought to initiate a conformational change of the membrane receptors, which brings about the activation of a regulatory enzyme located within the target cell membrane. This, in turn, might initiate other systems including glycolytic enzymes which provide the ATP needed to promote cell-granule membrane fusion and subsequently the release of histamine from the cell (Stanworth, 1973). Others suggest that the allergen may trigger an increased influx of Ca^{++} which in turn may set the histamine release into motion (Lawson et al., 1975).

Studies on the enzyme system in mast cells provide some interesting findings for the mechanism of histamine release. Based on experimental results from studies on human lung tissues, Austen and Valentine (1968) reported that treatment of rat mast cells with a rabbit anti-rat gamma globulin activated the serine esterase in the target cell. Treatment of sensitized human basophils with di-isopropyl phosphofluoride which inactivated serine esterase inhibited histamine release from the target cell upon challenging with the specific allergen (Austen and Brocklehurst, 1961). Lichtenstein (1968) later showed that this event only influenced the general metabolic pathway of the mast cell rather than the actual mechanism for histamine release. The serine esterase inhibitors are thought to block histamine release at the time of reaction between the allergen and IgE sensitized mast cell.

Lichtenstein (1973) showed that drugs which stimulate the alpha and beta adrenergic receptors on the mast cell/basophil membrane affect the release of histamine from the target cells. There is a correlation

between the level of cellular cyclic AMP and the amount of histamine released in the target cells. Drugs that activate adenyl cyclase, e.g. isoproterenol, or inactivate phosphodiesterase, e.g. methylxanthines, also exhibit an effect on histamine release. These findings led to a lot of suggestions for the mechanism of releasing the chemical mediators from mast cell.

The adenyl cyclase is now known to be located on the inner membrane of many types of cells. It is suggested that adenyl cyclase acts as the trigger site for a whole range of different hormones including histamine. By catalysing the formation of cyclic AMP from ATP, it may play an important role in conveying the message between the exterior and the interior of the cell. Cyclic AMP functions as a second messenger in acting on the appropriate intracellular site for a particular hormone which leads to the synthesis and secretion of other substances. It is proposed that histamine release could be triggered by the same mechanism. The binding of the allergen to the IgE-mast cell complex could trigger off a conformational change on the cell membrane. This would lead to an activation of the adjacent adenyl cyclase and hence the formation of cyclic AMP. Drugs which stimulate the beta adrenergic receptor, like isoproterenol and epinephrine, inhibit histamine release. The beta receptor is believed to be adjacent to the membrane-bound adenyl cyclase. An increased formation of cyclic AMP in mast cells via beta receptor stimulation suppresses histamine release. Norepinephrine which activates the alpha adrenergic receptor on the membrane decreases the formation of cyclic AMP, but enhances histamine release from the target cell. It is suggested that the site of the alpha adrenergic receptor is adjacent to that of the membrane-bound ATPase. Activation of the

ATPase results in the breakdown of ATP into ADP and inorganic phosphate; and hence depletes the source for cyclic AMP in the cell. Robison et al. (1967), however, proposed that the alpha and beta receptors could be regulatory subunits of the adenyl cyclase. The allosteric effect of these two subunits controls the formation of cyclic AMP.

Evidence has shown that the release of histamine from mast cell is also under the influence of prostaglandins, acetylcholine and cyclic GMP. The role of prostaglandins in anaphylaxis is uncertain. Some prostaglandins seem to inhibit the antigen-induced histamine release from sensitized basophils, while some seem to cause vasodilation and an increased vascular permeability. Acetylcholine stimulates the parasympathetic receptors which brings about histamine release. The reaction is believed not to be cyclic AMP mediated. Cyclic GMP, on the other hand, has an enhancing effect on histamine release. The relation between the cellular levels of cyclic AMP and cyclic GMP on the control of histamine release is not clear.

CHAPTER II

INTERACTION OF MAST CELLS WITH HOMOCYTOTROPIC ANTIBODIES AND ANTISERA TO MAST CELLS

1. Interaction of Mast Cells with Homocytotropic Antibodies

Introduction

IgE antibodies are frequently referred to as reaginic antibodies or reagins. These antibodies have both homocytotropic and heterocytotropic properties since they can interact with homologous target cells (mast cells and basophils) and target cells of closely related species, respectively. Thus, Ishizaka (1968) has shown that human IgE, which is homocytotropic in man, functions as a heterocytotropic antibody in monkeys but not in guinea pigs. The cytotropic function of IgE antibodies has been explored by means of the PCA methods in animals or the P-K test in man. Optimum reactions are obtained after a latent period of two or more days and passively sensitized skin sites remain sensitized for two weeks to months.

Homocytotropic antibodies have been demonstrated in mouse, rat, guinea pig, rabbit, dog, monkey and man. In general there are two major types of homocytotropic antibodies. The first group belongs to the IgG class of immunoglobulins. They are present in relatively high concentration in serum. Chemical studies reveal that they are stable to heat treatment and sulfhydryl reagents (Ishizaka et al., 1971). Optimum PCA reactions are obtained after a latent period of one or a few hours and passively sensitized skin sites remain sensitized for up to several hours. Mouse IgG1 (Mota et al., 1968), guinea pig IgG1a (Ovary et al., 1963) and rat IgG2a (Bach et al., 1971) antibodies are examples of this

category. The reaginic antibodies belong to the second type of homocytotropic antibodies. They are characterized by their sensitivity towards heat, as well as reduction and alkylation. Thus, they lose their cell fixing ability if they are heated at 56°C for over four hours, or if they are reduced (Ishizaka et al., 1969). They are present in very low concentration in serum (0.1 µg/ml) under normal condition. Their binding to the target cells is strong and they remain bound for a long period of time (Bach et al., 1971). Ishizaka et al. (1967) in their studies on the immune response to ragweed pollen in allergic subjects discovered that reaginic antibodies belong to a unique class of immunoglobulins. They called it the IgE class. The discovery of a monoclonal IgE protein from a myeloma patient (N.D.) by Johannson and Bennich (1967) contributed greatly to the understanding of the structure and function of IgE. Studies on papain digested fragments of IgE demonstrated that the unique determinants of this class were located in the Fc fragment, whereas the light chain determinants were confined to the $F(ab')_2$ fragment (Bennich and Johannson, 1968). Chemical studies revealed that IgE molecules also have the heavy and light chain structure as the other immunoglobulins. The molecular weights of these chains of IgE are 72,500 and 22,600, respectively. Studies on the amino acid sequence of ε chain revealed that it is longer than the heavy chain of the IgG class and like IgM has an extra domain in the constant region. Dorrington and Bennich (1973) demonstrated, by circular dichroism, that the C_3H and/or C_4H domain(s) of the ϵ chain are conformationally altered by heat treatment at 56°C. Since the cell fixing property of IgE is destroyed by this treatment, the authors postulated that these domains of the Fc region are involved in binding to target cells.

Receptors for the Homocytotropic Antibodies

The two types of homocytotropic antibodies are present in the body at the same time. Since they both bind to the same target cells and induce the same mediator release, it is of considerable interest to determine if they interact with the same receptors on the target cell surface and utilize the same biochemical pathway leading to the release of pharmacologic mediators. Bach et al. (1971) demonstrated that rat IgG_{2a} could inhibit the release of histamine from rat peritoneal mast cells treated with rat IgE. Stanworth and Smith (1972) reported that human immunoglobulins of the IgG_4 subclass had the capacity to bind to primate skin and to compete with human IgE in subsequent sensitization of the skin. The above inhibitions of IgE induced functions by IgG type homototropic antibodies can be due to steric hindrance exerted by the IgG molecules sitting on adjacent receptors rather than competition on the same site. Therefore, it is not clear as yet if they bind to the same receptor.

Receptors for Heterologous Reaginic Antibodies

The concept that reagins are incapable of interacting with target cells of distantly related species is largely based on failure to sensitize guinea pig for PCA reaction by human reagins (Ishizaka, 1968). Studies by several groups of workers show that heterologous sensitization is possible. Perelmutter (1970) et al. sensitized rat mast cells with human penicillin allergic sera. Gillman et al. (1972) using human reaginic serum or E myeloma protein induced histamine release from rat mast cells. Vijayanagar et al. (1974) demonstrated the same thing. However, there is also some evidence which indicates that sensitization of rat mast cells by human reagins is difficult to achieve (Plaut et

al., 1973 and Ishizaka et al., 1975). Recent findings by König and Ishizaka (1974) and Kulczycki et al. (1974) show that mouse IgE antibodies can bind to the surface of rat mast cells and basophils, and hence inhibit the binding of rat IgE antibodies to their homologous target cells. Prouvost-Danon et al. (1975) demonstrated cross reactivities between mouse and rat IgE with their heterologous mast cells. The data suggest not only that there is homology in the structure of the cytotropic regions of reagins from different species but also that these reaginic antibodies might interact with a common receptor, on the surface of their target cells.

Nature of the Receptors for IgE

Using radio-labelled anti-IgE and the technique of autoradiography, Ishizaka et al. (1970a) have provided the first direct evidence that IgE antibody is present on the surface of human basophil. Later, they applied the same technique to demonstrate the presence of IgE molecules on human skin mast cells (Ishizaka et al., 1971). Independent evidence for the presence of IgE on the surface of its target cell has been obtained by means of a rosetting technique (Wilson et al., 1971). Sullivan et al. (1971) demonstrated the same thing by using the method of ferritin labelling and electron microscopy. Bach et al. (1973) showed the specific binding of rat IgE to a cell free particle preparation from rat peritoneal mast cells. These findings suggest that human basophils and mast cells have receptors for IgE molecules. Treatment with anti-IgE antibodies results in degranulation of the human basophils and subsequently histamine release (Ishizaka et al., 1969). Thus, it has

become clear that the release of pharmacologic mediators from these cells is due to an Ag-Ab reaction on the surface of basophils and mast cells.

Interaction between IgE and the receptors

The concept that the Fc fragment of the IgE molecule is responsible for the cell fixing property of this immunoglobulin originated from indirect evidence given by Stanworth et al. in 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 P-K reaction induced by human reaginic serum on human skin. Ishizaka et al. provided the direct evidence by showing the binding of radioactive labelled E myeloma protein to the human basophil surface via the Fc portion of the molecule (1970b). It is now generally accepted that the receptor for IgE antibody on the target cell surface has affinity for the Fc region of the molecule.

The presence of surface receptors for complement, the Fc portion of the antibody molecule, aggregates of immunoglobulins and specific antigens has long been established in the lymphoid cell system by means of rosette formation, fluorescence labelling and autoradiographic techniques. One common phenomenon observed by various techniques upon demonstrating the presence of surface receptors is cap formation. Cap formation is also observed in guinea pig macrophages and basophilic leukocytes (Ferrarini et al., 1973). Sullivan et al. (1971) reported the phenomenon of capping on human basophils using anti-human IgE and a ferritin labelling technique. The distribution of ferritin on the basophil surface is temperature dependent, i.e. patch formation occurs at 0°C and capping at room temperature. Becker et al (1973) studied the

distribution of surface IgE on human basophils using fluorescence microscopy and immunoferritin electron microscopy. They demonstrated that the redistribution of IgE was dose, time, and temperature dependent. At low doses of anti-IgE no gross redistribution was observed. At higher doses patch formation and capping were seen. Rapid redistribution with internalization was observed at even higher doses. Cap formation was seen within 10 - 30 minutes when cells were incubated with high dose of anti-IgE at $37^{o}C$ and interiorization was observed when the incubation was prolonged (1.5 - 4 hours). They also showed that capping on human basophils required divalent anti-IgE. In other words, bridging of IgE on basophils is necessary for redistribution of these surface molecules. They further demonstrated that cap formation of surface IgE on atopic human basophils interacting with anti-IgE antibodies was not a prerequisite for subsequent histamine release.

The availability of neoplastic rat basophilic leukemia (RBL) cell (Eccleston et al., 1973) has enabled the study of surface distribution of IgE on basophils to a full extent. Kulczycki et al. (1974) showed the specific binding of rat IgE to these cells. Carson and Metzger (1974) demonstrated the surface redistribution of the receptor on cultured RBL cells preincubated with rat IgE. Redistribution of the receptor for IgE is energy dependent. Isersky et al. (1975) using the same cell line, estimated that there were up to 10^6 receptors for IgE per RBL cell. Sullivan et al. (1971) calculated that there were approximately 4×10^5 receptors per human basophil based on their ferritin labelling data. Recently, Conrad et al. (1975) estimated that there were 6×10^5 receptor sites per RBL and 3×10^5 per rat mast cell. Ishizaka et al. (1973) stated that there were $10^4 - 4 \times 10^4$ IgE molecules present on

human basophils and the total number of receptor sites for IgE per basophil upon saturation was up to $3 \times 10^4 - 10^5$. More recently, Ishizaka et al. (1975) estimated that the number of receptors on rat mast cells was in the range of $3 \times 10^5 - 8 \times 10^5$ per cell. Hence, the range number of receptors for IgE on the target cell is still waiting to be verified.

Ishizaka et al. (1973) found that cell bound radio-labelled IgE was replaceable by cold IgE; Therefore, they proposed that the binding of IgE to the surface receptor was a reversible process. They further estimated the value of the affinity constant for the interaction of human basophils and IgE to be of the order of $10^8 - 10^9$ M^{-1} and suggested that such a high affinity was indicative of a highly specific receptor for IgE on the surface of human basophils. Kulczycki et al. (1974) demonstrated that the binding between IgE and its specific receptor is indeed reversible and can be described by the following equation:

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

The association rate constant (k_1) between free IgE molecules and the surface receptors of RBL cells was 1.3×10^5 M^{-1} Sec^{-1}. The initial binding rate was dependent on the IgE concentration. The dissociation rate constant (k_{-1}) of the receptor-IgE complex was extremely low (1×10^{-5} - 1.35×10^{-5} sec^{-1}). The affinity constant for the binding was thus estimated to be $\simeq 10^{10}$ M^{-1}. Conrad et al. (1975) on studying the interaction between rat IgE and rat mast cell receptors, showed that the receptors were homogeneous based upon the results obtained from Scatchard plots. Their calculated value of the affinity constant for both rat mast cells and rat basophilic leukemia cells (10^9 M^{-1}) was

slightly higher than that reported by Ishizaka et al. (1973) and somewhat lower than that reported by Kulczycki et al. (1974).

Characterization of Target Cell Receptor for IgE

Attempts have been made by several groups of workers to isolate the receptors for IgE from the RBL cell and rat mast cell surface. Bach et al. (1973) first reported the binding of IgE to rat mast cell membrane particles. König and Ishizaka (1974) obtained cell free particles of rat mast cells by ultrasonication. Fractionation of these particles which had affinity for rat IgE by either gel filtration or ion exchange chromatography resulted in several fractions. Only one of these fractions retained the IgE binding activity. The enzyme, 5'-nucleotidase, was used as a membrane marker. The presence of such enzyme activity suggested that this 'active' fraction consisted of plasma membrane material of the rat mast cells. Further purification of this fraction resulted in a material which bound IgE and had a molecular weight of 2×10^6 daltons.

Carson et al. (1975) demonstrated that cultured rat basophilic leukemia cells shed a surface component at $4^{o}C$. This component could bind rat IgE. Gel filtration of the ^{125}I-IgE bound particles from the RBL cells resulted in a fraction which retained most of the radiolabels but had a molecular weight larger than the free IgE molecule. They claimed that this fraction consisted of IgE molecules, membrane components and receptors. The molecular weight of this complex was in the range of 10^6 daltons.

Conrad and Froese (1976) have obtained an IgE receptor preparation of much lower molecular weight (62,000). They labelled the rat

mast cell or RBL cell surface with ^{125}I using lactoperoxidase, incubated it with rat IgE and disrupted the cell membrane with detergent, NP-40. The radiolabelled membrane preparation was precipitated and then fractionated by gel filtration. A distinct fraction was obtained in repeated experiments either with rat mast cells or RBL cells. The appearance of this fraction was IgE dependent. It was suggested that this could be the receptor for IgE molecules or a component thereof. The NP-40 solubilized IgE-receptor complex was estimated to have a molecular weight of $3.5 - 5.5 \times 10^5$ daltons (Conrad et al., 1976). Hence, the molecular size of the receptor itself was in the order of $2 - 4 \times 10^5$ daltons. This isolated receptor component appeared to be smaller than the ones reported by König et al. (1974) and Carson et al. (1975). When this receptor-IgE complex (Conrad and Froese, 1976) was dissociated with sodium dodecyl sulfate (SDS) and urea and analyzed by SDS-polyacrylamide gel electrophoresis, a major band corresponding to a molecular weight of 62,000 was obtained. Based on these findings, (Conrad and Froese, 1976) proposed two possible models for the structure of the receptor for IgE. The first model suggested that only the 60,000 dalton subunits of a two component receptor complex was exposed at the plasma membrane of the mast cell and was thus labelled by ^{125}I. Therefore, the SDS-polyacrylamide gel electrophoresis revealed one single radiolabelled component. The second component not exposed to the cell surface and not labelled by ^{125}I could thus not be detected. The second model predicted that the receptor complex consisted of several identical monomers each having a molecular weight of approximately 60,000 (Conrad and Froese, 1976).

The suggestion that the receptor for IgE on mast cell/basophil surface is membrane component is duly acceptable based on

the above data. Studies on the chemical nature of the plasma membrane of other cell system, e.g. erythrocytes, suggest that the membrane component most likely consists of a mixture of protein, phospholipids and carbohydrates. Conrad and Froese (1976) reported that their 'receptor component' was sensitive to trypsin digestion indicating that the receptor molecule was at least in part, a protein. Bach et al. (1973) have shown that binding of IgE to the receptor site on rat mast cell surface was sensitive to the treatment of sialidase and phospholipase C. They suggested that sialic acid and beta-linked galactose residues play a role in the binding of IgE to mast cells. The role of phospholipids on the binding is not yet clear. It is plausible to suggest that the chemical nature of the receptor for IgE could possibly be composed of protein, carbohydrate and perhaps lipid.

2. ANTISERA TO MAST CELLS

Introduction

Antisera to mast cells have been produced by several groups of workers at various periods of time. Although there exist only a few reports on such antisera, the emergence of each anti-mast cell serum has contributed to the further understanding of the biological role played by mast cells.

Specificities of the Anti-Rat Mast Sera

The first report on an antiserum to rat mast cells was that produced by Smith and Lewis (1961). Rabbits were immunized intravenously with 7 doses of purified rat peritoneal mast cells of 3 - 5 x 10^6 cells per dose over a period of 12 days. The anti-mast cell activity of the rabbit sera was screened by the interfacial precipitin test. The

presence of anti-mast cell activity in the serum was observed as early as 3 to 5 days after the last injection. Highest activity was seen at 10 to 12 days after the last injection and was maintained for about one week. Exposure of the intact, living rat mesentery to the antiserum resulted in tissue mast cell damage with degranulation. Tissues from rats injected intraperitoneally with the antiserum were examined microscopically. At one day after the injection, all of the mast cells of the mesentery, abdominal skin, scrotum and ear were found ruptured and the histamine content in these tissues fell drastically. Mast cells of these tissues were completely destroyed and the level of histamine reached its minimum value at 7 to 10 days after the last injection. Repopulation of mast cells and replenishment of the histamine content of these tissues appeared 4 to 6 weeks later. Injection of the antiserum to rats also led to morphological changes in the tissues of the peritoneal cavity; shrinkage of the mesentery and shedding of parts of the capsules of the liver and spleen. The anti-mast cell activities of normal rabbit serum as well as a rabbit anti-liver cell serum were tested in the same way as was the anti-mast cell serum. Normal rabbit serum showed no effect on such activities. The damaging effects of the anti-liver serum, however, were indistinguishable from those of the anti-mast cell serum. The main drawback of this work is, therefore, the questionable specificity of the anti-mast cell serum. As mentioned before, the anti-mast cell serum had some effects on the liver tissue, therefore, it is conceivable that this antiserum may interact with cells other than mast cells. Unfortunately, the authors did not perform additional tests to clarify this point. In view of the fact that the anti-mast cell serum had not been absorbed with unrelated cells, and that it had indistinguishable

activities as the anti-liver cell serum, it is possible that the properties of the anti-mast cell serum were largely due to antibodies which were not specific for mast cell surface antigens.

Hogarth-Scott et al. (1971) observed that an anti-rat peritoneal cell serum significantly suppressed the immune response to Nippostrongylus brasiliensis infection in the rat. The antiserum was induced in rabbits injected intravenously or intraperitoneally with a mast cell enriched preparation of mixed rat peritoneal cells. The anti-peritoneal cell serum had an inhibitory effect on homologous PCA reactions induced by the nematode antigen and its specific antibodies. It also induced histamine release and degranulation from peritoneal cells in the presence of complement. It was proposed that this antiserum was directed against antigens on the mast cell surface. However, the antiserum in this case cannot be considered as mast cell specific since the rabbits were immunized with mixed peritoneal cells which include lymphocytes, macrophages, erythrocytes and mast cells. Furthermore, the surface antigens on mast cells presumably include rat species specific antigens, rat major histocompatiblity or Ag-B antigens, structural antigens that are unique to mast cells, receptors for complement components and for various cytotropic antibodies. Only antigens that are confined to the mast cell surface can be strictly considered as mast cell specific. The anti-peritoneal cell serum undoubtedly contained a mixture of antibodies some of which were directed against the species specific and histocompatibility antigens. Since histamine release in this case was dependent on the complement, any anti-rat cell serum would have induced

mast cell damages. Hence, the blocking of PCA reactions by the anti-peritoneal cell serum does not suggest the presence of RMC specific antibodies. Moreover, the fact that the antiserum was capable of suppressing the immune response to N. brasiliensis may indicate that the serum did contain antibodies directed against rat immunoglobulins.

Valentine et al. (1967) in their studies on mechanisms of histamine release have induced an anti-rat mast cell serum by immunizing rabbits with 12×10^6 rat mast cells emulsified with Complete Freund's Adjuvant via the foot pads. The anti-rat mast cell serum induced histamine release from normal rat mast cells in the presence of complement. The mediator release was cytotoxic since it was often accompanied by cell death detected by vital staining. Absorptions of the antiserum with different types of cells were carried out. The antiserum absorbed with sheep or rat erythrocytes retained its sensitizing effect on normal rat mast cells whereas absorptions with peritoneal cells or enriched mast cells removed all sensitizing capacity. A rat lymph node cell preparation free of mast cells was able to reduce the sensitizing ability of this antiserum to a moderate extent. The antiserum was shown to have no specific activity on unrelated antigens like Ficoll and rat serum protein. The specificity of the anti-rat mast cell serum was reinforced by immunofluorescent staining on the mast cell surface, demonstration of its effect on diminishing the peritoneal histamine content in rats injected with the antiserum and its ability to induce a skin reaction in normal rats specifically. Indeed, this the most elegant work among the other published data as far as the specificity of the anti-rat mast cell serum is concerned.

CHAPTER III

CHARACTERIZATION OF ANTISERA TO RAT PERITONEAL MAST CELLS

Introduction

Previous studies on antisera to rat mast cells (RMC) have focussed on the involvement of these antisera in the release of histamine from mast cells (Smith and Lewis, 1961, Valentine et al., 1967). Interaction between the rat mast cell surface and the anti-RMC serum was demonstrated by an immunofluorescent staining technique (Valentine et al., 1967). Absorption studies on the antiserum by these authors have been mentioned in the previous Chapter. The results suggest that it is possible to obtain a rather specific anti-RMC serum provided that appropriate absorptions are performed. However, these previous studies revealed no information on the nature of the mast cell antigens that reacted with the antisera. The characterization of the surface antigens of mast cells is of great interest in the field of allergy, especially since the presence of the receptor for IgE as a unique RMC surface entity is now known and demonstrable. It is conceivable that the serum used by Valentine et al. (1967) contained antibodies directed against the receptor for IgE, or even to IgE (both ε and L chains), since the serum was not absorbed with IgE or rat immunoglobulins. However, the authors were not concerned with this question and their results do not provide an answer even in the light of today's knowledge. Therefore, the aim of the present study was to establish if anti-RMC serum contains antibodies against the receptor for IgE. Such a study was made possible by the recent discovery of a rat monoclonal IgE (Bazin et al., 1974a) as well as the development of techniques which

make it possible to study the binding of IgE to mast cells directly (Kulczycki et al., 1974, Conrad et al., 1975). In addition the receptor for IgE on the rat mast cell surface has been partially characterized (Conrad and Froese, 1976). The availability of an antiserum to the receptor for IgE would be extremely important in the further characterization of this receptor.

Materials

Animals:

Female Lewis-Wistar rats, (retired breeders, 250 - 400 g), were purchased from Canadian Breeding Laboratories (Montreal, Quebec).

Female New Zealand albino rabbits (5 - 6 lbs) were obtained from Canadian Breeding Laboratories (Montreal, Quebec).

Buffers:

THM: Tyrode's buffer was prepared according to the formula of Kabat and Mayer (1961) and was modified as suggested by Bach et al. (1971) 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, California. This buffer was adjusted to pH 6.8 and is now referred to as THM.

THM/BSA: Bovine serum albumin (BSA) purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, was added to THM buffer to yield a concentration of 500 µg/ml. This buffer is referred to as THM/BSA.

Antisera:

Anti-sarcoma cell serum: This antiserum, a gift from Drs. J. Dalton

and S. Fujimoto of this Department, was produced in a rabbit immunized with methylcholanthrene induced sarcoma cells from Lewis-Wistar rats. Hereafter it will be referred to as anti-S serum.

Anti-rat IgE serum: A gift from Dr. D.H. Conrad, this rabbit anti-rat IgE serum had been prepared as described by Bazin et al. (1974b). It had been rendered specific for ε-chains by absorption with the immunoglobulins of normal rat serum.

Anti-rat immunoglobulin serum: This was a multispecific antiserum prepared by Dr. B.G. Carter of this Department .

Goat anti-rabbit immunoglobulin serum (GAR): This antiserum was purchased from Miles Laboratories, Kankakee, Illinois.

Ascitic Fluids:

IR-162: This ascitic fluid of Lou/Wsl rats bearing the IgE secreting IR-162 immunocytoma was a gift from Dr. Hervé Bazin of Louvain University, Brussels, Belgium (Bazin et al., 1974a).

IR-33: This was an ascitic fluid from rats bearing the IgG_{2a} secreting IR-33 immunocytoma. It is also a gift from Dr. Hervé Bazin (Bazin et al., 1974a).

Methods

1. Purification of rat mast cells

The procedures were 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 was 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 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 heparin (ICN Pharmaceuticals, Inc., Cleveland, Ohio) was injected into the peritoneal cavity of 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 THM/BSA buffer and cell counts were performed with a hemacytometer (American Optical Corporation). Mast cells were identified by staining with neutral red which formed a dry film on the cover slip of the hemacytometer (0.2% neutral red in ethanol). The initial suspension contained 5 - 10% mast cells. The 4 ml cell suspension was 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 spun at 200 x g for 2 minutes at $4^{o}C$. At the end of this time, the upper layer and cells at the interface were removed. The tube wall around the interface 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. Finally the cells were counted and 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% of the mast cells were recovered in the final preparation and the purity was between 85 - 90%.

2. Immunization and serum collection

A. Immunization:

Two groups of rabbits were injected with isolated rat mast cells either intravenously or subcutaneously. Mast cells (10×10^6) from 5 rats were washed extensively with THM after the Ficoll separation procedure. For subcutaneous injection, the cells were suspended in 1 ml THM and were emulsified with an equal volume of Complete Freund's Adjuvant (CFA), (Difco Laboratories, Detroit, Michigan). The mixture was injected subcutaneously onto the shaved lateral parts of the rabbit. About 30 days later, the animal was injected a second time with mast cells (5×10^6) incorporated in CFA. Third and fourth doses of the same strength as the second one were given about 14 days and 28 days respectively after the second injection. For the intravenous injection, 5×10^6 mast cells in 1 ml THM were injected via the marginal ear vein. Six such doses were given over a period of 14 days.

B. Serum collection:

Blood was obtained from the marginal ear vein and the serum was separated out by allowing the blood to clot, followed by centrifugation at 1000 x g for 20 minutes at $4^{\circ}C$. Preimmune sera were collected from the rabbits and were kept separately for each individual animal. From rabbits immunized subcutaneously in the presence of CFA, sera were collected one day prior to subsequent injections. From intravenously immunized rabbits, sera were obtained 7 days after the last injection.

C. Processing of sera:

All sera were inactivated by heating at 56°C for 30 - 60 minutes and were filtered through Millipore membranes (20μ) and stored in sterile vials at 4°C. Serum was withdrawn from the vial with a sterile needle and syringe when required.

When required, the immunoglobulins of sera was precipitated by adding an equal volume of cold saturated ammonium sulfate (SAS) dropwise at 4°C. The precipitate was washed twice with 50% SAS and centrifuged at 10,000 rpm for 20 minutes. It was dissolved in a small volume of phosphate buffered saline (PBS) and dialysed against several changes of PBS at 4°C. After dialysis, the sample was reconstituted to its original volume. The preparation was then stored in sterile conditions as mentioned above.

3. Isolation of cells

A. Preparation of 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 anesthetized 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.

B. Preparation of rat liver and kidney cells.

The organs were removed from exsanguinated rats, rinsed and the tissues were disrupted with a loosely fitting glass homogenizer. The dispersed cells were suspended in PBS and filtered through Pyrex glass wool. The preparation was washed in PBS and centrifuged at 200 xg for 10 minutes.

The washing procedure was repeated till the supernatant became clear.

C. Preparation of rat mast cells:

Mast cells were isolated from the peritoneal fluid as mentioned before. Ten rats were used in order to obtain 0.3 ml of packed mast cells.

D. Preparation of rat lymph node (LN) cells:

Cervical lymph nodes were removed from exsanguinated Lewis-Wistar rats, rinsed and the tissues dispersed as described for liver and kidney cells. The cells were suspended in THM/BSA supplemented with 10% fetal calf serum (FCS). The subsequent treatment was identical to that used with liver and kidney cells.

4. Preparation of immunosorbent

This was a slightly modified method to that reported by Cuatrecasas and Anfinsen (1971). Ten ml of washed, packed Sepharose-4B were activated with cyanogen bromide (150 mg/ml 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 50% SAS-precipitated rat gamma globulin (14.7 mg/ml) or the IgE rich globulin fraction (15.4 mg/ml) from rat ascitic fluid IR162 (see Materials). The coupling reaction was carried out at $4^{o}C$ for 24 hours. The immunosorbent was washed with PBS until the filtrate was free of protein and then it was transferred into a beaker. Any unreacted Sepharose-4B was inactivated by adding 50 ml of 0.05 M ethanolamine at $4^{o}C$ and the reaction was allowed to proceed for 30 minutes. The gel was then washed with PBS, followed by 200 ml of 0.2 M glycine-HCl buffer (pH 2.2) and PBS again till the filtrate remained at pH 7 and had no trace of protein as determined by its optical density at 280 nm.

5. Absorption procedures

A. Cells:

About 0.1 ml packed cells was used for each ml of diluted serum (1:10). The mixture was incubated at 37°C for 15 minutes with constant rotation. At the end of each absorption, cells were spun down at 200 x g for 10 minutes and were removed. At least three to five absorptions were carried out with each cell type. The serum was clarified by centrifugation at 50,000 x g for one hour at 4°C.

B. Immunosorbent:

The immunosorbent 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. Usually the serum was absorbed once with IgE rich globulins from ascitic fluid IR162 followed by rat immunoglobulins. The absorbed serum was then filtered and stored in sterile vials at 4°C for future use.

6. Activities of the anti-rat mast cell serum

A. Intradermal skin test:

This skin test was based mainly on the modified technique of Valentine et al. (1967). The dorsal skin of an anesthetized rat was

shaved and skin sites were marked. About 0.05 ml 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. The diameter of each blue spot was measured twice to get the mean value. The color intensity was recorded in some experiments. Any spot having a mean diameter of less than 5 mm was regarded as a negative result. The titre of a serum was referred to as the highest serum dilution that still gave a positive skin reaction.

B. The Chromium-51 (^{51}Cr) cytotoxicity test:

The cytotoxicity 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 (FCS) (Grand Island Biological Company) and were labelled with 100 µci of $Na_2(^{51}Cr)O_4$ (Amersham-Searle Company, Arlington Heights, Illinois) at 37°C for 30 minutes. Sera to be tested were diluted with the same buffer directly on the tissue culture plate (Microtest II, Falcon Plastics, Oxnard, California). The cells were washed after labelling. In the case of lymph node cells, the cells were washed and spun down at 200 x g for 5 minutes. The washing was repeated for another 4 times. When mast cells were used as the target cells, they were layered gently on top of 1.5 - 2 ml heat inactivated FCS and spun down at 200 x g for 5 minutes. The cell pellet was resuspended in the medium and centrifuged. The washed cells which were then labelled with ^{51}Cr and suspended in 10 ml of medium (to yield a

concentration of 10^6 per ml). To 0.1 ml of serum in each well of the microtiter plate, 0.1 ml of a cell suspension (1×10^5 cells) was added, followed by 0.1 ml of complement (diluted 1:10). Guinea pig complement was used when lymph node cells were the target cells. Fresh rabbit serum served as the source of complement for the cytotoxicity test with mast cells. The plate was sealed with a piece of microtiter plate sealer (Cooke Engineering Company, 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 condition 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 percentage of specific ^{51}Cr release which was calculated with the following formula:

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

C. Degranulation test:

Purified rat mast cells (5×10^5) in a 0.1 ml suspension were incubated with 0.1 ml of antiserum in the presence or absence of 0.1 ml diluted rabbit complement (1:10) at 37°C for 30 minutes. Mast cells were stained with 0.25% toluidine blue in ethanol and were examined and

counted under the microscope. The results were expressed as % of degranulation [= (number of cells degranulated/total number of cells) x 100].

D. Binding of ^{125}I-IgE and ^{125}I-IgG$_{2a}$

a. Preparation of ^{125}I-IgE and ^{125}I-IgG$_{2a}$

Rat monoclonal IgE purified as described by Conrad et al. (1975) was available in the laboratory. It was labelled according to McConahey and Dixon (1966) using chloramine T. Purified IgE (100 µg in 0.05 ml of PBS) was labelled with one mCi of carrier-free ^{125}I (Amersham-Searle, Arlington Heights, Illinois) in the presence of 0.45 ml isotonic 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 isotonic 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 dialysed versus 2 litres of THM for 12 hours. 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/ml with THM/BSA and was stored in aliquots at -70^{o}C. The specific activities of the labelled IgE was in the range of 2 - 6 x 10^{6} cpm/µg. The counting was done in a Beckman gamma counter (Model 300, Beckman Instrument Company, Fullerton, California) with a counting efficiency of 55% for ^{125}I. Partially purified rat monoclonal IgG$_{2a}$ was kindly provided by Mr. R. Helm of this laboratory and it was labelled in an identical

manner to IgE. The specific activity of the labelled proteins was established.

b. Procedures for immunoprecipitation

The immunoglobulin fraction of the rabbit serum to be tested was adjusted to a concentration of 8 optical density units (O.D.)/ml with PBS. To 0.015 ml of the above solution, 0.95 ml of PBS and 0.05 ml of ^{125}I-IgE or ^{125}I-IgG$_{2a}$ were added and the mixture was incubated at 37°C for 30 minutes. Goat anti-rabbit immunoglobulin (GAR) (0.025 ml) was added to the mixture and the incubation was carried on for another 30 minutes. These conditions which yielded complete precipitation of rabbit immunoglobulins had been established for a given batch of GAR by Dr. D.H. Conrad of this laboratory. Precipitation was allowed to proceed at 4°C for 4 - 6 hours. The precipitate was transferred to a clean tube and washed four times with PBS by centrifugation at 250 x g for 10 minutes and its radioactivity was measured. Normal rabbit immunoglobulins and rabbit anti-rat IgE antibodies were used in place of the antiserum as controls. The results were recorded in cpm and were expressed as the concentration of IgE precipitable by the corresponding serum.

E. Inhibition of the interaction between IgE and rat mast cells:

The procedure was the one developed by Kulczycki et al. (1974) and modified by Conrad et al. (1975). Purified rat mast cells, 5 x 10^5 in 0.1 ml of THM/BSA, were incubated with 0.75 ml of antiserum (i.e. anti-RMC or anti-S) 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, 0.15 ml of ^{125}I-IgE (1 µg/ml) was added into each tube and incubation was

continued for another 120 minutes. The cell suspension was layered gently on top of 1.5 - 2 ml of heat inactivated FCS and centrifuged at 250 x g for 5 minutes. The supernatant was discarded and the tube with its cell pellet was assayed for its radioactivity. Cell pellets obtained from mast cells incubated with ^{125}I-IgE in the absence of antiserum were used as controls for the maximum binding of IgE. The radioactivity of ^{125}I present in these cell pellets were then determined. The average value obtained from these controls became the arbitrary 100% of IgE binding. Each result was recorded in cpm and was expressed as percentage of inhibition of IgE binding [(cpm of experimental result/cpm of maximum IgE binding) x 100].

7. Characterization of mast cell surface antigens reacting with the anti-rat mast cell serum

A. Surface labelling:

Mast cells were iodinated according to the lactoperoxidase method as described by Kennel et al. (1973) and modified by Conrad and Froese (1976). Purified rat mast cells (5 x 10^6 in 0.5 ml of isotonic PBS) were labelled with 500 µci of carrier-free ^{125}I (or one mCi of carrier-free ^{131}I purchased from New England Nuclear, Boston, Massachusetts) 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 and centrifugation at 250 x g for 5 minutes. The cells were resuspended in 1 ml THM/BSA and spun through a layer of FCS as described before. Cells were washed once more with THM/BSA before disruption in 1 ml of 0.5% Nonidet P-40 (NP-40) (Shell Chemicals,

England) in PBS (NP-40/PBS). After a 15 minute incubation at 37°C, the soluble material was isolated by centrifugation at 10,000 rpm for 15 minutes in a Sorvall RC2-B centrifuge (Ivan Sorvall Incorporated, Newtown, Conneticut). The supernatant was then dialysed versus NP-40/PBS for 12 hours.

B. Precipitation of cell surface antigens:

The solubilized, dialysed extracts of 5 x 10^6 mast cells were divided into several aliquots. Surface components present in each of these aliquots were either precipitated with the anti-RMC serum, the absorbed anti-RMC serum, rabbit anti-rat IgE serum or normal rabbit serum in the presence of goat anti-rabbit immunoglobulin serum. The precipitations were performed under optimal conditions (see Results and Discussion). The immunoprecipitate was solubilized by the addition of 0.1 - 0.2 ml of Buffer A (see 6C) followed by incubation at 37°C for 3 hours. All samples were dialysed against Buffer B overnight.

C. Polyacrylamide gel electrophoresis:

(i) Buffers -

Buffer A: 2% sodium dodecyl sulphate (SDS), 9M urea in 1 M Tris buffer, pH 8.5.

Buffer B: 0.01 M phosphate buffer, pH 7.2, containing 0.1% SDS and 0.5 M urea.

Buffer C: 0.1 M phosphate buffer, pH 7.4, containing 1% SDS in 5 M urea.

(ii) Method -

The samples were analysed according to the methods 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 Buffer C

and 2.5 ml of a 20% acrylamide in 0.5% methylene-bisacrylamide solution was deaerated and mixed with 0.5 ml of a freshly prepared 2% ammonium persulfate solution, 5λ of N,N,N',N'-tetramethylethylenediamine 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 harden. A drop of tracking dye (0.05% Bromphenol 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. Buffer B was carefully layered on top of each sample to fill the tubes. The upper and the lower reservoirs of the electrophoresis apparatus were filled with Buffer B. 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 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. When both ^{125}I and ^{131}I were present in the sample, the ^{131}I window of the 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) in the following manner.

$$\left[{}^{125}I \text{ counts after correction for spill over} = \text{total counts of the } {}^{125}I \text{ channel} - \text{total counts in the } {}^{131}I \text{ channel} \times \left({}^{131}I \text{ counts in the } {}^{125}I \text{ channel} / {}^{131}I \text{ counts in the } {}^{131}I \text{ channel}\right)\right].$$

All results were expressed graphically by plotting cpm versus fraction number starting from the top of the gel.

Results and Discussion

1. Immune response to rat mast cells

Five rabbits were immunized subcutaneously with rat mast cells in

Freund's Complete Adjuvant and three were immunized intravenously. One rabbit from each group died of laboratory infection during the course of immunization and their sera were discarded. The rest of the animals remained in good health. Sera collected from these animals after immunization were screened for their anti-rat mast cell activity. Several simple tests were tried out in the beginning in order to establish a rapid and easy method for screening the antibody activity. These included the precipitin ring test as described by Smith and Lewis (1961), a simple agglutination test modified from the method by Campbell et al. (1970) and a skin test for anti-mast cell activity described by Valentine et al. (1967). When the first two tests were tried out on the antisera, both failed to detect any anti-rat mast cell activity unless a large amount of purified rat mast cells was used. Unfortunately, the mast cell enrichment procedure is a tedious process and it does not meet the requirement for a rapid screening method. Therefore, the skin test was used. Valentine et al. (1967) reported that their anti-rat mast cell (anti-RMC) serum could induce a typical skin reaction, similar to the PCA reaction, 10 minutes after it was injected into the rat skin. The procedure was repeated with the anti-RMC sera produced during the course of this study. Evan's Blue was injected intravenously into the rats, 10, 13, 15, 17, 20 and 25 minutes, after the injection of the antiserum. It was found that when the dye was injected 10 or 13 minutes after the antiserum injection, nonspecific bluing reactions were obtained in the skin sites even when only buffer or saline instead of antiserum was injected. The nonspecific blue spots observed on the above sites were most likely due to a transient, slight skin irritation caused by the needle. The nonspecific reaction disappeared

if the dye was injected at a later time, e.g. after 15 minutes. However, if the period was extended beyond 20 minutes, the diameters of the blue spots would decrease indicating that the intensity of the reactions had diminished. In fact, no reaction could be seen if the dye was administered 25 minutes after the antiserum injection. Based on these findings, it was determined that the optimal time for anti-RMC serum to induce a specific skin reaction was between 15 to 17 minutes. Three to four rats were used for the skin test each time. The skin reaction fluctuated from one animal to another. A set of optimal results is shown in Fig. 1. Some rats would give a positive response to solutions like PBS, saline or THM while some did not respond at all, even to antisera of very low dilution. Therefore, the results were taken from rats that responded naturally, and averaged. They were expressed either as the antibody titre and/or the size and colour intensity of the skin reaction.

The antisera were titred in the following manner. Sera from the same animal but of different bleedings (i.e. preimmune, bleedings after the first, second injections and etc.) were diluted and tested for their efficacy in inducing the skin reaction. Table I shows some of the results obtained from one of the immunized rabbits, rabbit B. A 1:10 dilution of preimmune serum gave a blue spot of 5 mm while the same serum at higher dilution was unable to induce the reaction. The presence of a small amount of natural species specific antibodies (rabbit against rat) in the normal serum could have accounted for the effect on rat skin mast cells. The skin activities of the antiserum became stronger as the number of immunizing injections increased. For instance, an 1:10 dilution of an antiserum after the second bleeding (obtained after

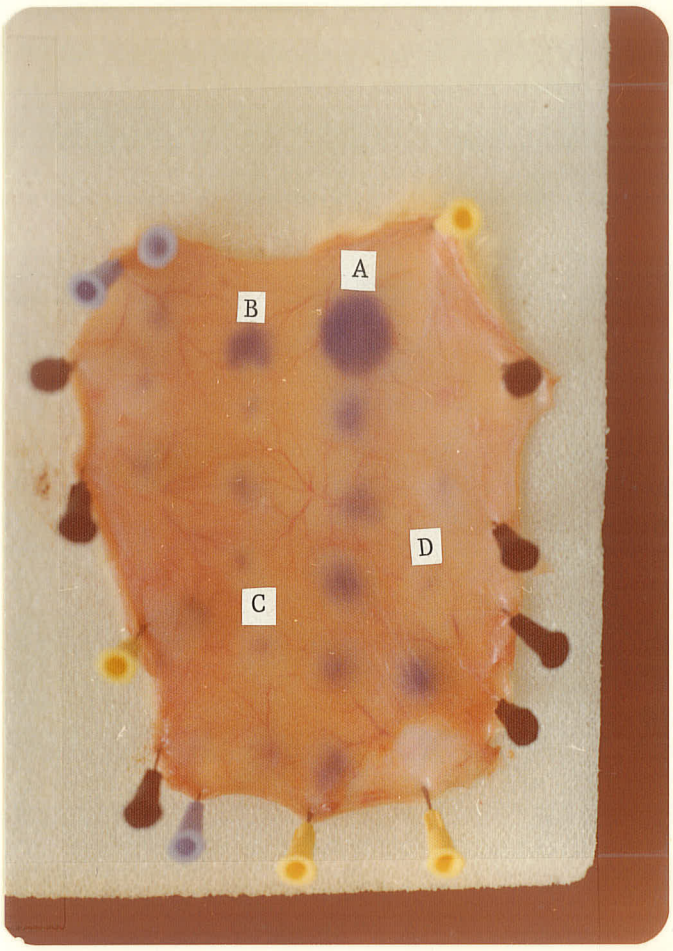

A
B
C
D

Figure 1. A typical example of the skin reaction induced by the anti-rat mast cell serum

Spot A: anti-RMC (1:50)

Spot B: pre-immune serum (1:10)

Spot C: pre-immune serum (1:100)

Spot D: saline

TABLE 1

SKIN ACTIVITY OF AN ANTI-RMC SERUM DURING THE COURSE OF IMMUNIZATION[a]

	Serum dilution	Rat A diameter (mm)	Rat B diameter (mm)	Average diameter (mm)
Pre-immune serum (first bleeding)	10	5.0	5.0	5.0
	20	---[b]	---	---
	40	---	---	---
	80	---	---	---
Antiserum (second bleeding)	10	12.0	8.0	10.0
	20	11.0	5.0	8.0
	40	8.0	5.0	6.5
	80	---	---	---
Antiserum (third bleeding)	10	15.0	16.0	15.5
	20	10.0	15.0	12.5
	40	9.0	13.0	11.0
	80	8.0	12.0	10.0
	100	6.0	7.0	6.5
Controls	saline	---	---	---
	PBS	---	---	---

[a] The serum was obtained from Rabbit B.

[b] Blue spots of a diameter below 5 mm were considered to represent a negative result.

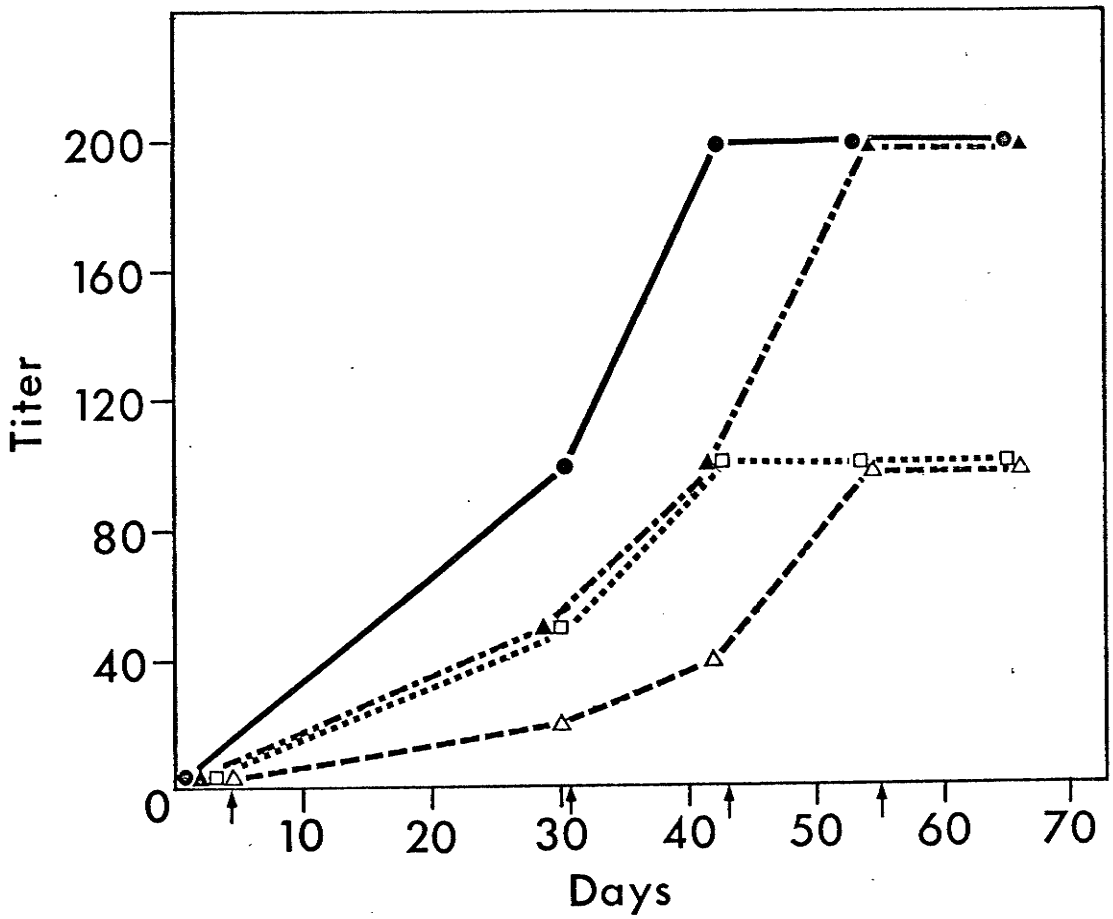
the first injection) gave a skin reaction of 10 mm while serum (1:10) from the third bleeding (obtained after the second injection) gave a skin reaction of 15.5 mm. The antibody titre also increased during the course of immunization from a titre of 40 in the second bleeding to 100 in the following one.

When antisera collected from all the immunized rabbits were tested for their abilities to induce the skin reaction, it was found that rabbits immunized with rat mast cells intravenously did not give high titred sera as compared to those being injected subcutaneously in CFA. Among the latter group of animals, rabbit E and rabbit I, gave better responses than the rest. Their sera induced the skin reaction at a serum dilution of 1:200 (Fig. 2) and of the two, rabbit I was the better responder since it yielded a serum which, after the first injection had a titre of 1:100. Hence, unless otherwise specified, serum from rabbit I giving a skin reaction titre of 200 was used for most of the subsequent studies. Results obtained by immunizing rabbits with mast cells intravenously are not shown in Figure 2. Titres of the two surviving rabbits never exceeded 40. This method of immunization was therefore discontinued.

One of the physiological features of mast cells is to degranulate in the presence of cell-bound IgE and specific antigen. Valentine et al. (1967) have shown that the anti-RMC serum that they produced could only cause histamine release from mast cells in the presence of fresh normal rabbit serum. Based on this finding, the anti-RMC produced in this study was also tested for its effect on mast cell degranulation. It was found that this anti-RMC serum had a stronger degranulation effect on mast cells in the presence of fresh normal rabbit serum

Figure 2. The immune response to rat mast cells.

Rabbits were immunized with purified rat peritoneal mast cells in complete Freund's adjuvant. The arrows indicate the time when injections were given. The titre of the sera was established by skin test in normal rats as described under Materials and Methods. Rabbit A (Δ— — —Δ), rabbit B (□·····□), rabbit E (▲—·—▲) and rabbit I (●——●) were bled just prior to injections.


200
160
120
80
40
0
Titer
10
20
30
40
50
60
70
Days

than in its absence. However, further study on the effect of anti-RMC serum on mast cell degranulation was discontinued because this method was not sensitive enough to titrate the antibody activity and reproducibility was poor.

2. Characterization of anti-RMC sera:

Common surface antigens present on most types of rat cells are the species specific and the transplantation (histocompatibility) antigens. Tissue or cell type specific antigens are found only on the surface of certain cells, e.g. mast cell defined antigens are present only on mast cells. The mast cell surface incorporates all the above antigens. Hence, the anti-RMC serum may have contained antibodies directed against non-mast cell specific antigens. In order to remove such nonspecific antibodies from the anti-RMC serum, it was necessary to find a good source of cells which carried the common rat cell antigens but excluded all mast cell specific antigens. Tissue homogenates are generally used for this purpose. In most mammalian species, including the rat, spleen cells and LN cells obtained from tissue homogenates are the best source for the common antigens but they both inevitably contain mast cells in their connective tissues. Instead, rat erythrocytes, kidney and liver cells were used for this purpose. All these contain a minimum number of mast cells (Rizzetto and Doniach, 1973). Anti-RMC serum was absorbed three times with these different cell types separately. At the same time, the preimmune serum was absorbed the same way with rat erythrocytes. The capacities of these cell types to remove nonspecific antibodies from the anti-RMC serum was tested on the basis of cytotoxicity to rat lymph node (LN) cells. The surface of LN

cells contains a lot of common antigens. Rat LN cells were labelled with ^{51}Cr and used as target cells for sera absorbed with the cell types listed above. Figure 3 shows that rat erythrocytes were effective in removing natural antibodies from the normal preimmune rabbit serum, but less so in absorbing out most of the antibodies against rat common antigens from the anti-RMC serum. In contrast, Valentine et al. (1967) showed that absorption of their anti-RMC serum with rat or sheep red cells had no effect on the capacity of the antiserum to sensitize rat peritoneal cells for histamine release by normal rabbit serum. These results suggest that the serum of rabbit I did contain natural antibodies against some rat cell surface antigens. Such antibodies must have been absent from the serum used by Valentine et al. (1967). Both kidney and liver cells were more effective than erythrocytes in removing the cytotoxic activity of the antiserum for rat LN cells. (Fig. 3), with liver cells having a slight edge over kidney cells. It was then decided that rat liver cells would be used for future absorption procedures since they are available in larger quantity than kidney cells and since they removed rat common antigens slightly more efficiently than the latter. Before the characterization of the anti-RMC serum proceeded any further, it was important to find out whether the above absorptions would deplete the skin activity of the antiserum. Hence, the absorbed sera were tested for their capacities to induce the skin reactions in rats. It was found that the absorptions did diminish the size of the skin reaction but not the Ab titres (Table 2). For instance, the unabsorbed anti-RMC serum gave a blue spot of 18 mm diameter at a 1:10 dilution while the anti-RMC absorbed with liver cells gave a diameter of 14 mm at the same dilution in the same rat. On the other hand,

Figure 3. Cytotoxicity for rat LN cells of anti-RMC (rabbit E) absorbed with various cell preparations.

Unabsorbed (●——●), absorbed with erythrocytes (Δ- - - -Δ), absorbed with kidney cells (▲- - - -▲), absorbed with liver cells (O- - - -O), pre-immune serum (□·—·—□), pre-immune serum absorbed with erythrocytes (●——■).

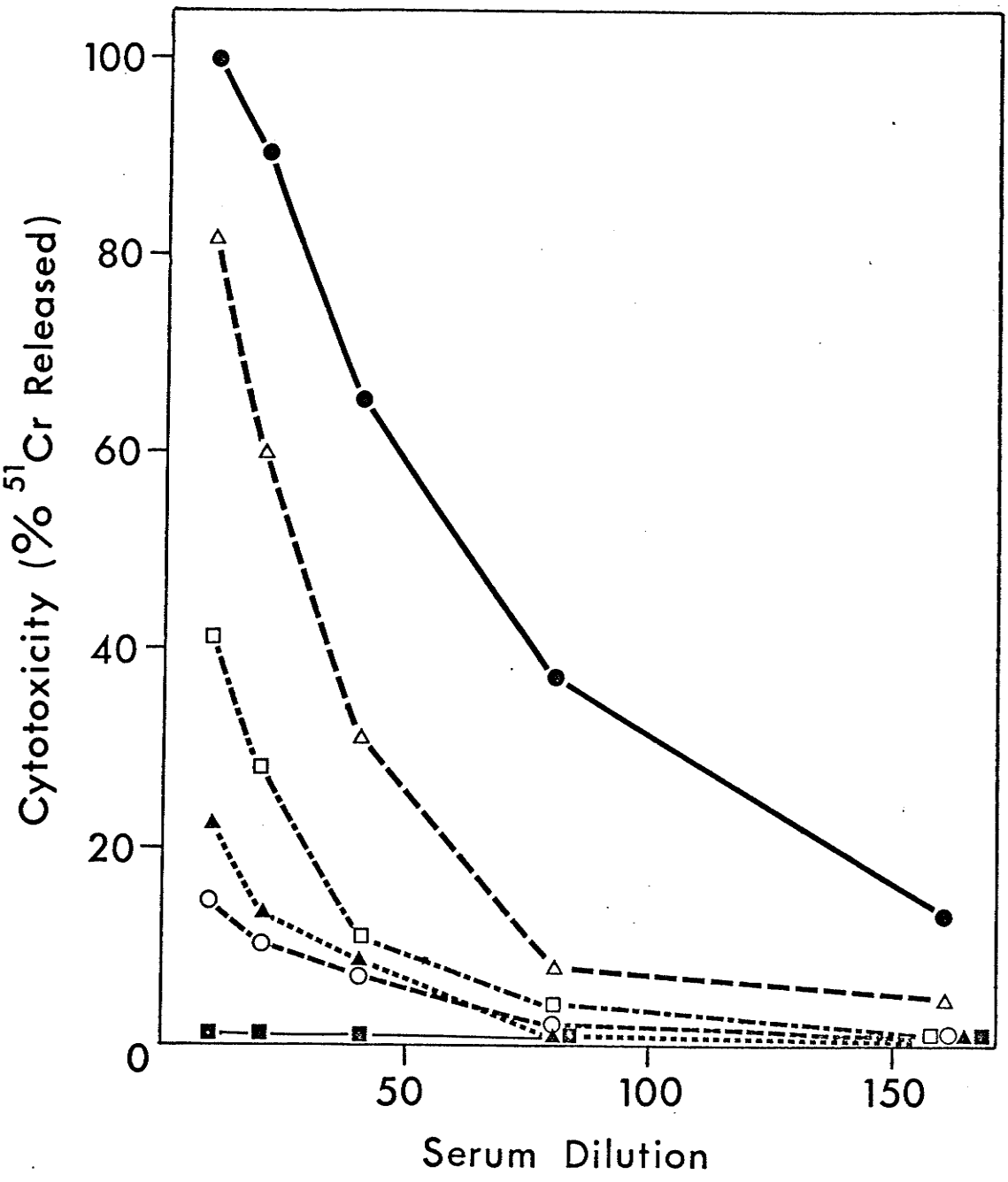

100
80
60
40
20
0
Cytotoxicity (% ^{51}Cr Released)
50
100
150
Serum Dilution

TABLE 2

SKIN ACTIVITIES OF ANTI-RMC ABSORBED WITH DIFFERENT RAT CELL PREPARATIONS[a]

Serum \ Serum Dilution	Rat 1				Rat 2			
	Diameter (mm)				Diameter (mm)			
	1:10	1:50	1:100	1:200	1:10	1:50	1:100	1:200
Anti-RMC	18.0	12.0	8.0	6.0	16.0	11.5	8.0	7.0
Anti-RMC$_{red}$[b]	13.0	8.0	6.0	5.0	13.0	9.0	6.0	5.0
Anti-RMC$_{kid}$[c]	15.0	9.0	5.0	5.0	15.0	10.0	7.0	5.0
Anti-RMC$_{liv}$[d]	14.0	10.0	7.0	5.0	15.0	11.0	7.0	5.0
NRS[e]	5.0		---		6.0		---	
NRS[e]$_{red}$[b]	----		---		----		---	

[a] Each ml of 1:10 diluted serum obtained from rabbit E was absorbed three times with 0.1 ml of packed cells.

[b] It indicates that serum was absorbed with rat erythrocytes.

[c] It indicates that serum was absorbed with rat kidney cells.

[d] It indicates that serum was absorbed with rat liver cells.

[e] Normal rabbit serum was obtained from rabbit E before immunization.

both the unabsorbed and absorbed antisera could induce a skin reaction at a serum dilution of 1:200. As was the case with rabbit B (Table 1), the preimmune serum (NRS) of rabbit E also showed marginal skin activity, which could be absorbed with rat erythrocytes (Table 2). These observations suggest that the preimmune sera from both rabbits contained some natural antibodies to rat antigens (see also below).

The removal of the non-mast cell specific antibodies from the anti-RMC serum did not eliminate its ability to induce the skin reaction. The above results suggested that, the skin reaction was a specific test for the anti-rat mast cell activity. Whether or not the skin reaction was of a cytotoxic nature could not be resolved through these results. Valentine et al. (1967) had demonstrated that their anti-RMC serum released histamine only in the presence of complement. Therefore, the skin reaction observed by these authors was also most likely complement mediated.

The next step for characterization of the anti-RMC serum was to investigate if it contained anti-rat immunoglobulin activity. Mast cells are known to have homocytotropic antibodies attached to their surface under normal physiological conditions. These antibodies, mainly of the IgE class, are antigenic and may still remain bound to the mast cell surface after the purification process. In order to remove any antibodies against these molecules, which could possibly have been present, the anti-RMC serum was absorbed with an IgE rich fraction of rat immunoglobulin coupled to Sepharose 4B, followed by another absorption with whole rat immunoglobulins, coupled to the same supporting medium. In order to ensure that the absorption was complete, the binding of rat IgE and rat γ globulin by the absorbed

anti-RMC serum was determined by the immunoprecipitation method described under Materials and Methods. Monoclonal rat IgE purified from the ascitic fluid IR-162 and a partially purified IgG_{2a} preparation obtained from the ascitic fluid IR-33 (see Materials) were labelled with carrier-free ^{125}I separately. The amount of immunoprecipitates formed between the labelled antigens and the anti-RMC as well as the absorbed anti-RMC sera in the presence of goat anti-rabbit immunoglobulins is shown on Table 3. The data suggested that anti-RMC did not contain a significant amount of anti-rat Ig and particularly anti-IgE antibodies. The absorptions with rat IgE and rat Ig had decreased the binding of these antigens by the absorbed anti-RMC serum to levels equivalent to those obtained with normal rabbit serum. These values most likely represent the nonspecific trapping of radioactive proteins in the precipitates.

In order to render the anti-RMC serum as specific as possible, it was routinely absorbed with an IgE rich fraction of rat immunoglobulins, normal rat immunoglobulins and five times with rat liver cells. This antiserum, referred to as anti-RMC_{abs}, was tested for its cytotoxic effect on rat lymph node (LN) cells and rat mast cells separately. The cytotoxicity test using LN cells as target cells was performed in order to investigate if the absorbed antiserum still contained antibodies against rat antigens common to several tissues, whereas the latter test with mast cells was used to establish the specificity of this absorbed antiserum for RMC. An unrelated serum, anti-sarcoma cell (anti-S) serum, (see Materials), was used as a control. This anti-S serum was absorbed with liver cells the same way as the anti-RMC serum. It will be referred to as anti-S_{abs}. Preimmune serum absorbed with rat

TABLE 3

BINDING OF ^{125}I-IgE or ^{125}I-IgG$_{2a}$ BY DIFFERENT ANTISERA

Antiserum[a]	Amount of ^{125}I-IgE bound[c] (µg)	Amount of ^{125}I-IgG$_{2a}$ bound[c] (µg)
Anti-rat IgE	0.0390	
Anti-rat Ig		0.0243
Anti-RMC	0.0015	0.0052
Absorbed anti-RMC[b]	0	0.0018
NRS	0.0015	0.0021

[a] 0.12 optical density units of the immunoglobulin fraction of each antiserum was used for the precipitation

[b] This antiserum was absorbed with IgE rich rat immunoglobulins followed by whole rat immunoglobulins.

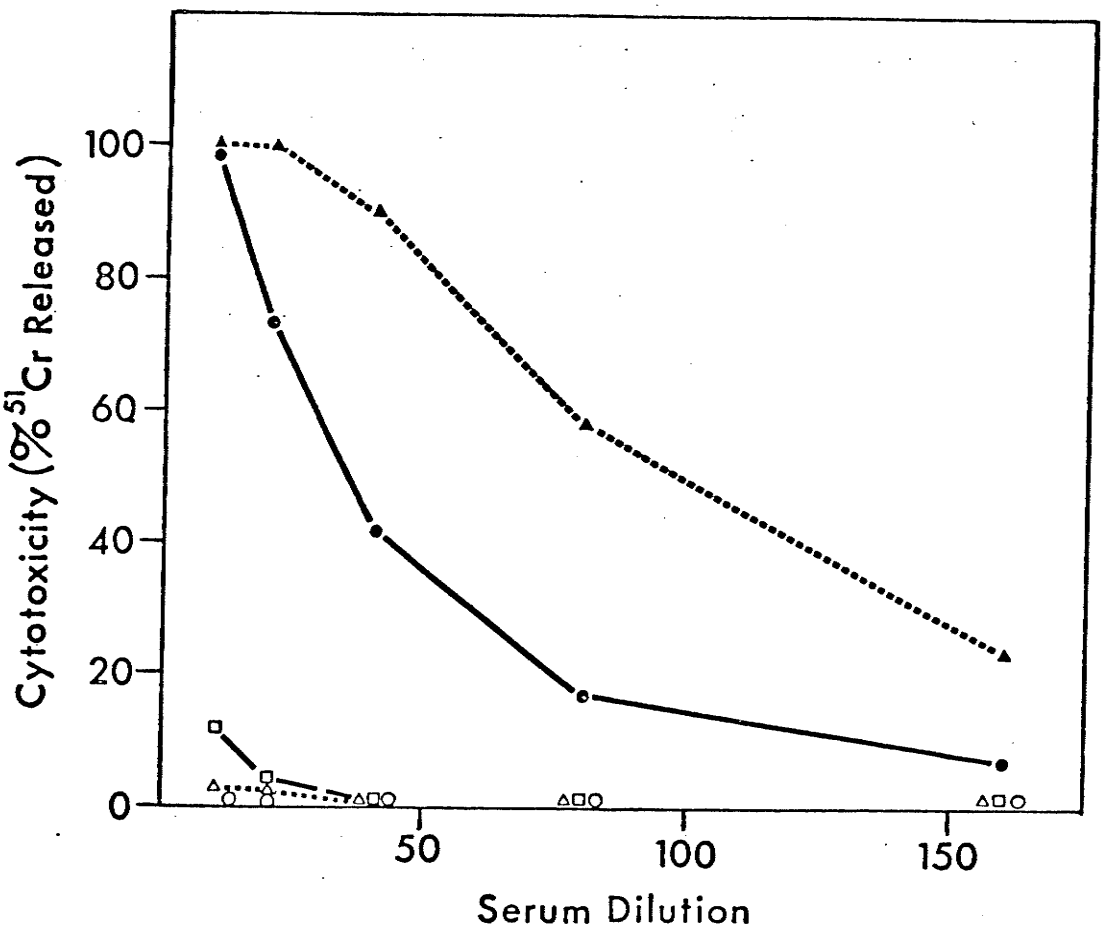
[c] The background ^{125}I-activity has been subtracted from each result

erythrocytes was used as another control. Figure 4 shows that the anti-S serum was more cytotoxic to rat LN cells than the anti-RMC serum. For instance, 50% of ^{51}Cr release was achieved by the anti-S serum at a dilution of 1:100 whereas a higher serum concentration, a dilution of 1:35, of anti-RMC was required in order to accomplish the same cytotoxic effect. The anti-S serum probably contained more antibodies against the rat LN cell surface antigens than the anti-RMC serum. After liver cell absorptions, both antisera (anti-RMC_{abs} and anti-S_{abs}) exerted a minimum cytotoxic effect on the target cells indicating that the absorptions were sufficient enough to remove cytotoxic antibodies against rat LN cell surface antigens. The next step was to investigate if these antisera, now depleted of the above antibodies, still killed rat mast cells specifically.

A detailed study for the cytotoxic release of ^{51}Cr from rat mast cells was performed since no such work had been reported so far. It was found that the uptake of ^{51}Cr by rat mast cells was comparable to that by LN cells. Both cell types picked up the radiolabel very efficiently. However, mast cells tended to have a higher value of spontaneous release than the LN cells. Mast cells are generally more fragile than other kinds of cells. Experimental conditions including repeated centrifugations for the washing procedure after the radiolabelling process could cause spontaneous cell lysis of mast cells. Usually, these conditions did not affect the viability of other cell kinds, e.g. LN cells, thymus cells and mastocytoma cells (P815 from DBA/2 mice). In order to minimize the risk of spontaneous lysis, the labelled mast cells were spun through a layer of heat inactivated FCS followed by one wash with the buffer. This method was adapted from

Figure 4. Cytotoxicity of various antisera for rat LN cells as measured by the percentage of ^{51}Cr released specifically.

Rat LN cells ($1x10^5$) were incubated with guinea pig complement and different dilutions of: anti-RMC (●——●), anti-RMC_{abs} (Δ- - -Δ), anti-S (▲- - -▲) anti-S_{abs} (□——□) and pre-immune serum absorbed with rat erythrocytes (○——○).


Cytotoxicity (% ^{51}Cr Released)
100
80
60
40
20
0
50
100
150
Serum Dilution

the one described by Conrad et al. (1975). It was found that the modified washing procedure did reduce spontaneous cell lysis and that unbound radiolabel was removed just as efficiently as by the repeated washing method. Valentine et al. (1967) had shown that fresh normal rabbit serum, as a source of complement, was more effective in inducing histamine release from rat mast cells sensitized with rabbit anti-RMC serum than was human or guinea pig serum. In the present study it was also found that while normal guinea pig serum was effective for the cytotoxic killing of P815 mastocytoma cells by an anti-mastocytoma cell serum and of rat LN cells by the anti-RMC serum, it was not effective when mast cells were used as target cells. On the other hand, normal rabbit serum was found to be active for the killing of rat mast cells by the anti-RMC serum. A typical set of ^{51}Cr release data from both LN and RMC cells is shown in Table 4. Figure 5 shows that 50% of ^{51}Cr release was achieved by the anti-RMC serum at a dilution of 1:130. The anti-RMC_{abs} required a higher serum concentration, i.e. a dilution of 1:15, for the same cytotoxic effect on rat mast cells. The result indicated that absorptions of the anti-RMC serum did remove a large amount of nonspecific antibodies which could have accounted for the high cytotoxic effect of the unabsorbed anti-RMC serum. After the absorptions, the antiserum mainly contained mast cell specific antibodies which no longer killed rat LN cells but it was still cytotoxic to rat mast cells in the presence of complement. The specificity of the test was supported by the results obtained with control antisera. The anti-S serum was cytotoxic to rat mast cells in the presence of rabbit complement although the effect was less potent than that of the anti-RMC serum. To achieve a lysis of 50%, the anti-S serum could

TABLE 4

CPM OBTAINED FROM BOTH LN AND RMC CELLS DURING THE ^{51}Cr CYTOTOXICITY TEST

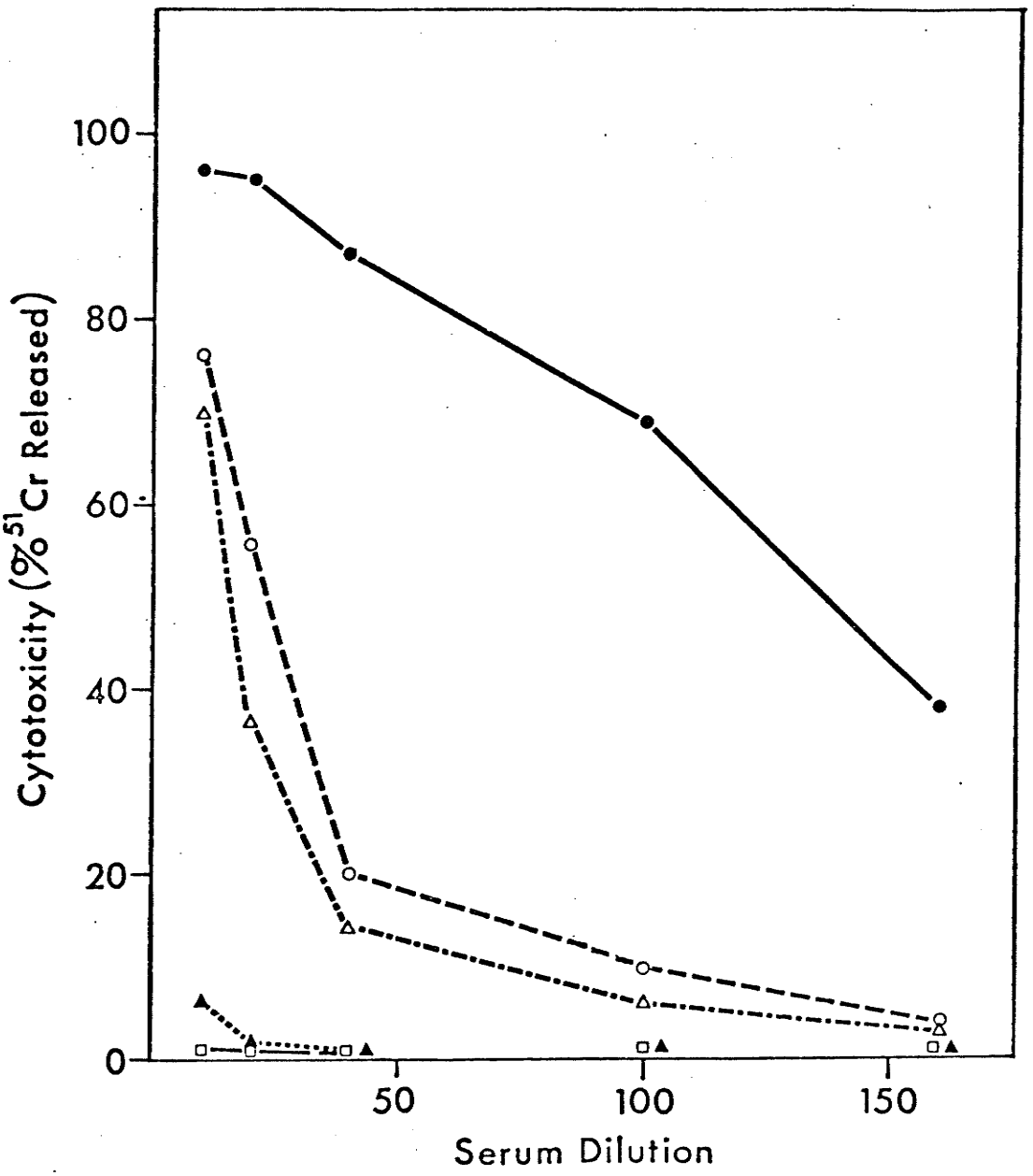
Cell type	LN[a]	RMC[b]
^{51}Cr uptake/10^5 cells (cpm)	15,588.0	69,829.0
Max. Release (cpm)	2,650.0	10,661.5
Spon. Release (cpm)	260.0	784.5
Release due to anti-RMC (1:10) (cpm)	2,300.0	9,894.0

a Lymph node cells (10^7) were labelled with 100 µci ^{51}Cr and washed five times with THM/BSA supplemented with heat inactivated FCS. Guinea pig complement was used.

b Rat mast cells (10^7) were labelled with 100 uci ^{51}Cr and centrifuged through a layer of heat inactivated FCS. The cell pellet was washed once more with THM/BSA supplemented with heat inactivated FCS. Rabbit complement was used.

Figure 5. Cytotoxicity of various antisera for RMC as measured by the percentage of ^{51}Cr released specifically.

RMC ($1x10^5$) were incubated with rabbit complement and different dilutions of: anti-RMC (●——●), anti-RMC_{abs} (Δ- - -Δ), anti-S (O- - -O), anti-RMC absorbed with RMC (▲- - -▲), and anti-S_{abs}/ pre-immune serum absorbed with rat erythrocytes (□——□).


100
80
60
40
20
0
Cytotoxicity (% ^{51}Cr Released)
50
100
150
Serum Dilution

only be diluted 1:30. These findings confirmed that non-mast cell specific antibodies play a role in the cytotoxic release of ^{51}Cr from rat mast cells. These nonspecific antibodies were completely absent from the anti-S_{abs} serum as well as from the absorbed normal rabbit serum. By contrast, anti-RMC_{abs} still retained some antibodies capable of lysing RMC in the presence of complement. Only when anti-RMC was absorbed with RMC did the serum lose its cytotoxic properties for rat mast cells. The result further confirmed the specificity of the antiserum. The above data suggested that the antibodies now present in the anti-RMC_{abs} serum were specifically directed against the mast cell surface antigens or at least that this serum contained antibodies to antigens present on RMC but absent from rat liver and LN cells. Such antibodies were removable by absorption with rat mast cells.

In order to further test the interaction of the various sera with rat mast cells, an investigation on the inducibility of the skin reaction by the above sera was performed by injecting all these sera (at a dilution of 1:20) into the skin of the same rat. The results are shown in Table 5. The diameter of the skin reaction induced by the anti-RMC_{abs} serum was in sharp contrast to the one absorbed with purified rat mast cells alone, since the latter induced no specific skin reaction. In other words, specific antibodies which were capable of inducing the skin reaction were completely removable by absorption with rat mast cells but only slightly so with rat liver cells and rat immunoglobulins. The slight skin reactivity of the anti-S serum represented the presence of nonspecific antibodies cytotoxic to the skin mast cells in the presence of complement. These nonspecific antibodies were completely removable by liver cell absorption. The skin test was not as

TABLE 5

SKIN ACTIVITY OF VARIOUS SERA

SERUM (1:20)	Rat 1		Rat 2		Rat 3	
	Diameter (mm)	Color intensity	Diameter (mm)	Color intensity	Diameter (mm)	Color intensity
Anti-RMC	17.0	++++	15.0	++++	14.5	++++
Anti-RMC_{abs}	15.0	++++	13.0	++++	11.5	++++
Anti-RMC_{abs}(RMC)	-	-	-	-	-	-
Anti-S	7.0	+	8.0	+	7.5	+
Anti-S_{abs}	-	-	-	-	-	-
PBS	-	-	-	-	-	-
Tyrode's/BSA	-	-	-	-	-	-

reproducible and quantitative as the ^{51}Cr cytotoxicity test owing to the difficulties mentioned and discussed in the previous section. Nevertheless, it still provided additional support pointing to the specificity of anti-RMC_{abs}. These results confirmed that anti-RMC_{abs} contained antibodies against antigens present on the mast cell surface, but not on the surface of either liver cells nor LN cells.

After the specificity of the anti-RMC_{abs} had been established, the next step was to see if it contained antibodies directed against the receptor for IgE on the mast cell surface. As mentioned in Chapter II, RMC have about 3×10^5 receptors for IgE on each cell (Conrad et al., 1975). Therefore, it is possible that the abundant receptor molecules could contribute to the antigenic determinants of the mast cell surface. Hence, the anti-RMC serum may have included some antibodies directed against these receptor molecules. In order to investigate such a possibility, the anti-RMC serum was tested for its ability to compete with radio-labelled IgE for the receptor on the mast cell surface as measured by the inhibition of ^{125}I-IgE. If the antiserum could inhibit the binding of IgE to rat mast cells, then it is possible that it may have contained antibodies against the receptor for IgE. On the other hand, no inhibition in the binding assay would demonstrate that the receptor was not involved. Figure 6 shows that anti-RMC sera obtained from two different immunized rabbits had inhibitory effects on the IgE binding to mast cells. In order to establish the specificity of the test, additional antisera, including the absorbed anti-RMC_{abs}, the anti-RMC serum absorbed with rat mast cells, the anti-S and the absorbed anti-S_{abs}, were assayed at the same time. The results are shown on Figure 7. Both the anti-S and the anti-RMC sera inhibited

Figure 6. Inhibition of IgE binding to RMC by two anti-RMC sera: anti-RMC_I (from rabbit I), anti-RMC_E (from rabbit E) and normal rabbit serum.

RMC ($5x10^5$) were incubated with 0.75 ml of the appropriate antiserum at a given dilution. After 30 minutes, 0.15 ml of ^{125}I-IgE (1µg/ml) was added. Binding was measured after 150 min.

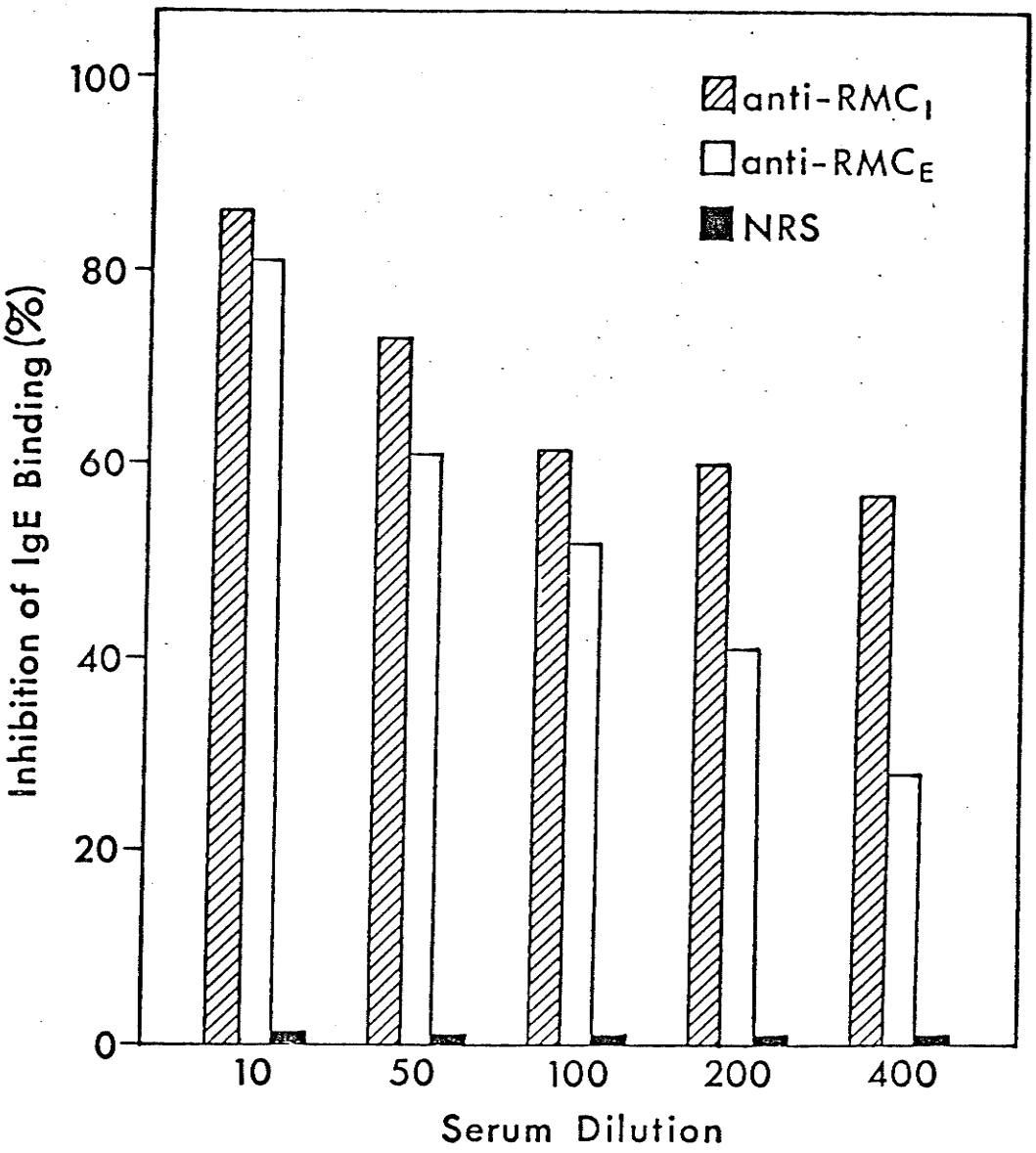
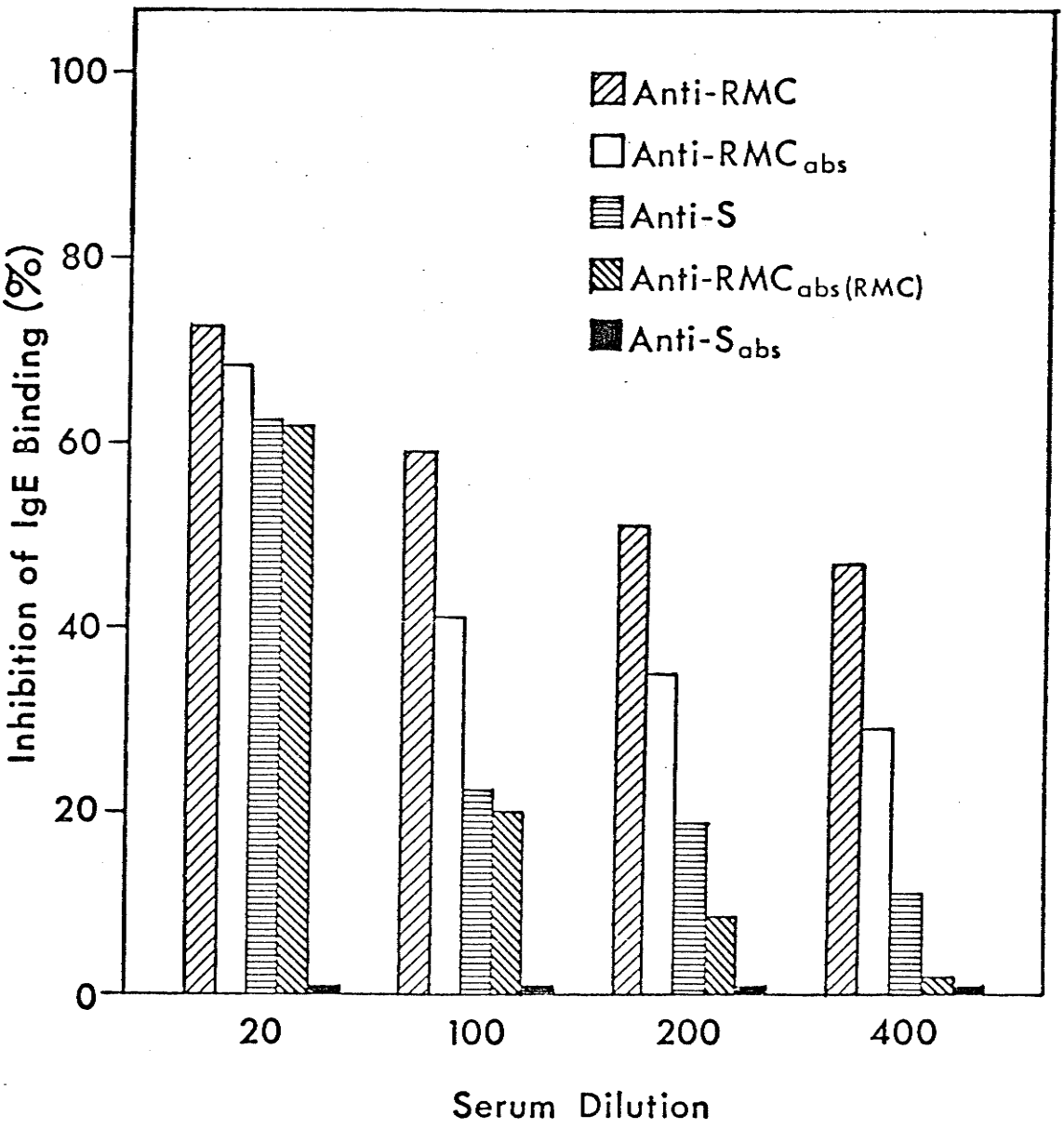

100
80
60
40
20
0
Inhibition of IgE Binding (%)
anti-RMC$_I$
anti-RMC$_E$
NRS
10
50
100
200
400
Serum Dilution

Figure 7. Inhibition of IgE binding to RMC by various antisera.

RMC ($5x10^5$) were incubated with 0.75 ml of the appropriate antiserum. After 30 min., 0.15 ml of ^{125}I-IgE (1µg/ml) was added. Binding was measured after 150 min.


100
80
60
40
20
0
Inhibition of IgE Binding (%)
Anti-RMC
Anti-RMC$_{abs}$
Anti-S
Anti-RMC$_{abs(RMC)}$
Anti-S$_{abs}$
20
100
200
400
Serum Dilution

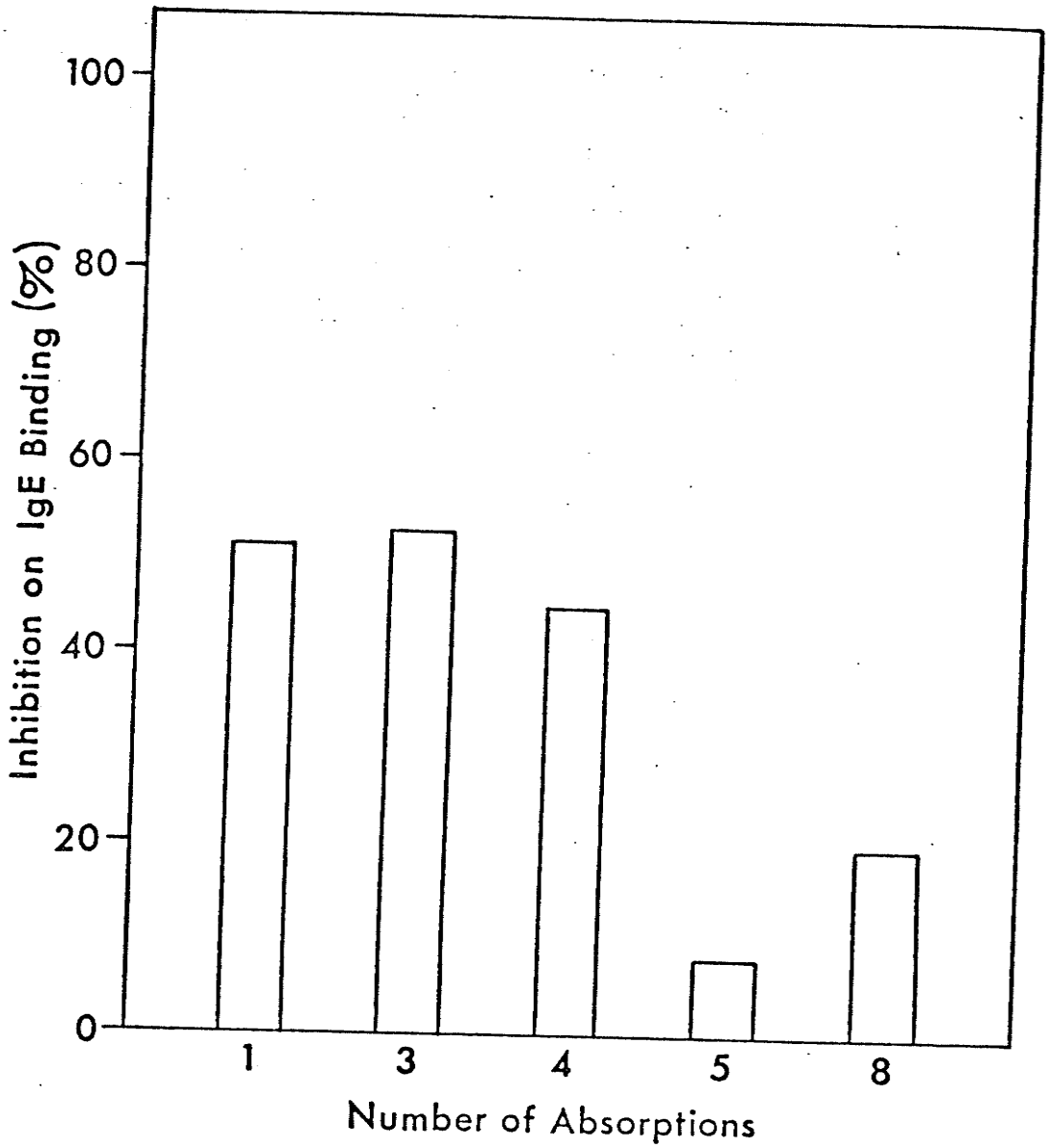
the binding of IgE to mast cells. It is obvious that the blocking activity exerted by the anti-S serum was not specific for the IgE receptor since it is very unlikely that sarcoma cells have a receptor for IgE on their surface and thus cannot induce the formation of antibodies to such a receptor. The data obtained with anti-S suggest that an inhibition of IgE binding does not necessarily indicate a direct interaction between the antibodies and the receptor for IgE. For inhibition of IgE binding by anti-S serum to take place, the antigens interacting with this antiserum must reside in close proximity to the receptor for IgE. However, it is interesting that anti-S_{abs} serum no longer inhibited the binding of IgE to mast cells while the anti-RMC_{abs} serum still maintained such activity. The anti-RMC serum could inhibit 50% of the total IgE bound to mast cells at a serum dilution of 1:260. The same level of inhibition was achieved by the anti-RMC_{abs} serum at a higher concentration, i.e. at a dilution of 1:60. The inhibition of IgE binding by RMC thus turned out to be the most sensitive test for measuring the activity of both anti-RMC and anti-RMC_{abs}. Even at a dilution of 1:400 both sera still significantly inhibited the binding of IgE. In contrast to the results obtained with the ^{51}Cr release assay and the skin test, anti-RMC serum absorbed with purified rat mast cells [anti-$RMC_{abs(RMC)}$] possessed an inhibitory activity in the binding assay (Fig. 7). These results were rather surprising particularly since anti-RMC absorbed five times with RMC, was no longer cyototoxic to RMC, nor did it induce a skin reaction. However, in view of the fact that the inhibition of IgE binding was a much more sensitive assay than the other two tests, the possibility that the anti-RMC serum had not been sufficiently absorbed, had to be considered. Therefore, additional absorptions were

performed. However, as seen in Figure 8, these did not lead to a further reduction of the inhibitory activity. As a matter of fact this activity seemed to increase again after the 8th absorption. The most likely explanation for this behavior was that surface receptors might have been shed from mast cells during the absorption procedures and remained mixed freely with the antibodies in antiserum. Once in excess over their specific antibodies, these receptors would combine with the ^{125}I-IgE and thus inhibit its binding to receptors on the surface of RMC. This effect would be expected to increase after a number of absorptions.

Since neither the preimmune serum nor the anti-S serum had an inhibitory effect on the binding of IgE to mast cells, the blocking activity of the anti-RMC_{abs} serum can be considered to be RMC specific. There are several possibilities to explain the inhibitory properties of anti-RMC_{abs}. The antiserum may have contained antibodies that reacted with antigenic determinants situated adjacent to the receptor for IgE. Binding of antibodies by these antigens would render the receptor inaccessible due to steric hindrance to the IgE molecules present in the medium. On the other hand, having antibodies directed against the receptor for IgE is an obvious explanation for the inhibitory effect of the anti-RMC_{abs} serum. Immunoglobulin E molecules cannot reach their receptors which are now covered by their specific antibodies. Only when these antibodies decrease in number, as would be the case at high serum dilutions can the IgE molecules bind to the cell surface. However, the third option of the absorbed anti-RMC serum having antibodies against both the receptor for IgE and their nearby antigenic determinants should not be excluded. More analytical methods would be required in order to

Figure 8. Inhibition of IgE binding to RMC by anti-RMC absorbed with RMC .

Anti-RMC (1:100) was absorbed several times with RMC. For each absorption, 1 ml of 1:10 diluted antiserum was absorbed with 0.1 ml of packed cells. The experimental conditions were the same as in Fig. 6 & 7.


100
80
60
40
20
0
Inhibition on IgE Binding (%)
1
3
4
5
8
Number of Absorptions

distinguish among the above possibilities.

3. Characterization of mast cell surface antigens reacting with the anti-RMC serum.

The lactoperoxidase technique for labelling of cell surface components with radio-active iodine has provided a convenient method for studying the nature of cell surface antigens. Based on this method Conrad and Froese (1976) were able to identify the receptor for IgE among the rest of the mast cell surface components. In order to prove directly that the anti-RMC serum had antibodies against the receptor for IgE, it was necessary to establish an identity between the receptor and the mast cell surface antigens interacting with the antiserum. First of all, the mast cell surface materials were iodinated as described in the section on Methods. The radiolabelled materials could be measured readily and hence, their fate during subsequent treatment could be followed easily. The mast cells were solubilized with Nonidet P-40 (NP-40) and were then allowed to interact with different anti-sera. The antigen-antibody complex was then precipitated by using the sandwich system described above, under Methods. The precipitates were solubilized and analysed by SDS polyacrylamide gel electrophoresis. The fate of surface components labelled with ^{125}I, starting with the solubilized and dialysed cells is illustrated in Table 6. The cpm obtained from solubilized cells represent only the ^{125}I associated with macromolecules, since any labelled low molecular components, most likely unsaturated membrane lipids (Kennel et al., 1973), had been removed by dialysis. It was found that about 4 - 5% of the total radiolabelled macromolecular mast cell surface materials extracted with NP-40 was precipitable with the anti-RMC serum whereas the amount that interacted with

TABLE 6

RECOVERY OF ^{125}I cpm ASSOCIATED WITH RMC SURFACE MACROMOLECULES UPON PRECIPITATION WITH ANTI-RMC SERA[a]

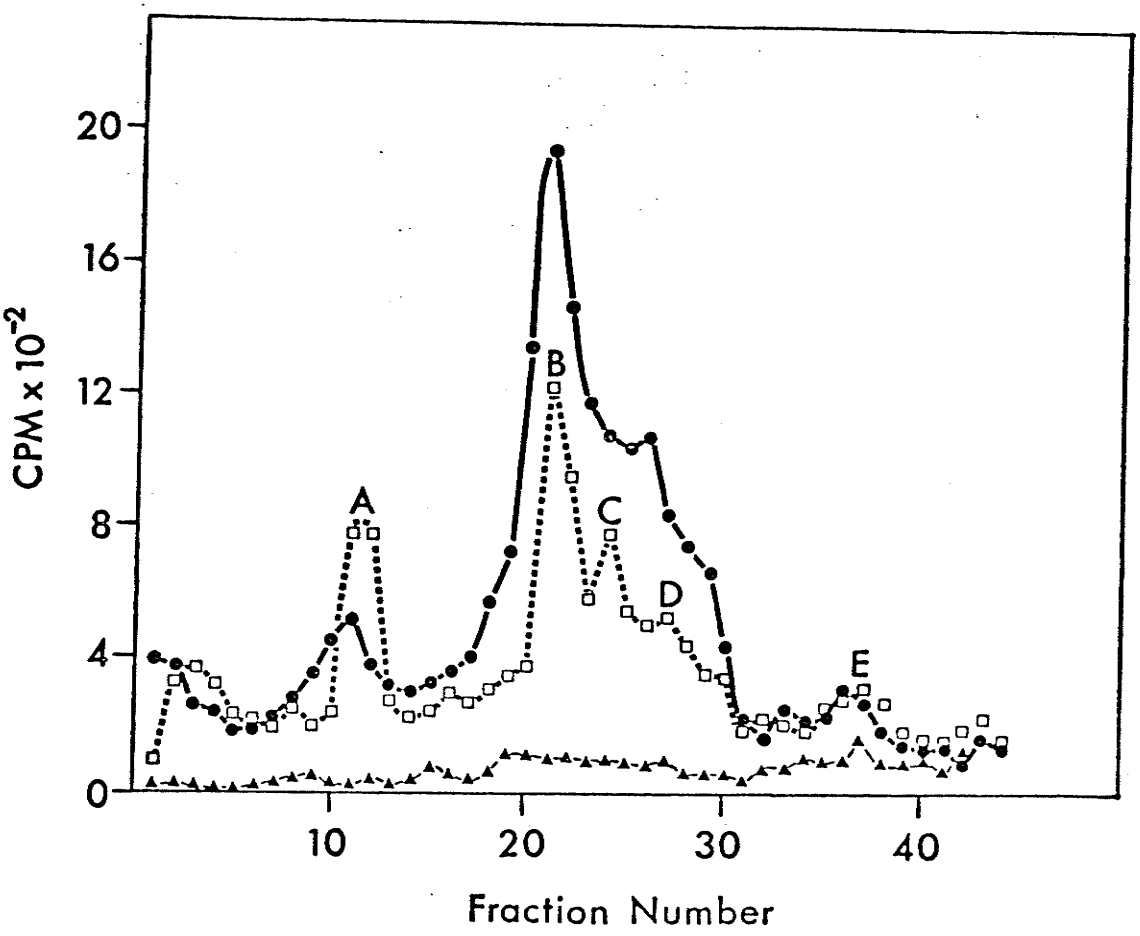
Precipitating serum	CPM of solubilized cells	CPM precipitated (before dialysis)	CPM precipitated (after dialysis)
Anti-RMC	932,916	40,529	28,370
Anti-RMC$_{abs}$	932,781	31,966	23,088
NRS	924,408	8,198	5,257

[a] Purified rat mast cells (5×10^6) were surface labelled with ^{125}I and solubilized with 1 ml of PBS/NP-40. After dialysis against PBS/NP-40, the surface materials were divided into 4 aliquots, the radio-active content of each was measured. Three of these aliquots were treated with different sera and the cpm in the precipitates formed were measured. These precipitates were then solubilized in SDS-urea buffer and dialysed against Buffer B as described under Methods. Again the radio-active content of each sample was determined.

the anti-RMC_{abs} serum was even less, around 3%. The precipitates were solubilized in a buffer containing SDS and urea (Buffer A page 42). Before the solubilized precipitates could be analysed by electrophoresis, it was necessary to dialyse them against a SDS-buffer (see Methods). Table 6 shows that about 30% of the total radio-active content present in the precipitate in each case was removed after the dialysis. When these precipitates were analysed by SDS polyacrylamide gel electrophoresis, the nature of the antibody-associated mast cell surface antigens was revealed (Figure 9). Different surface antigens were segregated into various zones along the gel column according to their molecular weights. Hence, materials that migrated fast towards the bottom of the gel had low molecular weights (10,000 - 20,000) daltons according to Conrad and Froese (1976). Those having higher molecular weights (100,000 - 200,000 daltons) would stay near to the top of the gel. By plotting the numbers of counts per minutes (cpm) versus the 2 mm-gel fractions, different peaks could be visualized. Each peak represented a group of surface antigens having a particular molecular weight. The number of peaks and their positions formed a constant pattern for each system. For instance, the same pattern appeared everytime when the mast cell surface antigens interacted with the anti-RMC serum. By comparing the patterns obtained from different precipitates (Figure 9), characteristic peaks representing the mast cell surface antigens interacting with their specific antibodies could be identified. It should be pointed out that the total number of cpm precipitated by the anti-RMC_{abs} serum was less than that brought down by the anti-RMC serum (Table 6). However, the antigens precipitated by the absorbed

Figure 9. SDS-polyacrylamide gel electrophoretic analysis of RMC surface components precipitated by anti-RMC sera.

Surface components precipitated by anti-RMC (●——●), by anti-RMC$_{abs}$ (□- - -□) and by normal rabbit serum (▲——▲).


20
16
12
8
4
0
CPM x 10⁻²
A
B
C
D
E
10
20
30
40
Fraction Number

anti-RMC serum yielded a pattern with more clearly defined features. Five distinct peaks could be observed in the precipitate obtained with the anti-RMC_{abs} serum whereas only four appeared in the precipitate brought down with the anti-RMC serum. These five peaks appeared at fixed positions along the gel column and probably represented different groups of antigens present on the mast cell surface. They were designated alphabetically according to the order of their appearance in the gel pattern. Peak A was a slow migrating band which appeared near to the top of the gel, between fractions 10 - 13. Peaks B, C and D had medium electrophoretic mobilities and were observed between fractions 19 - 22, 23 - 25 and 26 - 29 respectively. Peak E was a fast moving band which migrated near to the bottom of the gel, between fractions 35 - 39. As can be seen, when normal rabbit serum was used in the sandwich precipitation system, hardly any peaks were observed upon polyacrylamide-gel electrophoresis. The significance of the peak in fraction 37 will be discussed below. Even though the two patterns of Figure 9 are not drastically different from each other, it is obvious that the absorption of the anti-RMC serum had resulted not only in quantitative but also in qualitative changes.

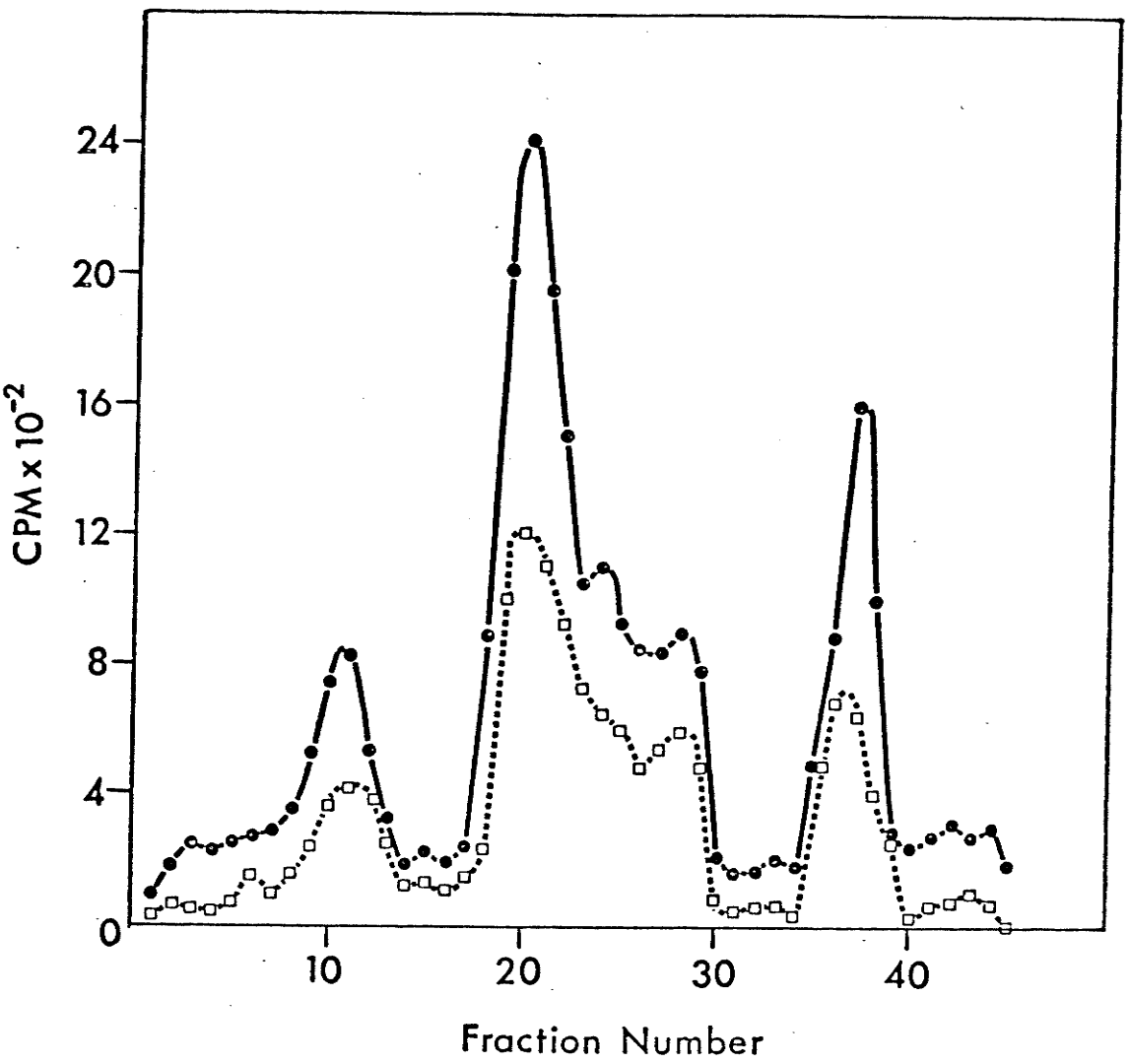
Before the sandwich system could be applied, the optimal amounts of antigen (rabbit immunoglobulins) and antibody (goat anti-rabbit immunoglobulins) had to be determined in order to precipitate all of the rabbit immunoglobulins (anti-RMC and anti-RMC_{abs}). Once the correct amount of the antibody was determined, it was kept constant and the concentration of the antigen (rabbit Ig) was adjusted to maximum precipitation for different systems. For instance, about three times as much material, in terms of optical density units, of anti-RMC_{abs} as anti-RMC had to be

used in order to obtain an optimum precipitation. Serial absorptions of the anti-RMC serum, as mentioned previously, had diluted the antiserum about three fold. However, the optical density of this serum had not decreased drastically due to non Ig materials introduced during the various absorption steps. Therefore, by adding three times as much anti-RMC_{abs}, the amount of rabbit immunoglobulins precipitable by the same concentration of goat anti-rabbit immunoglobulins was about equal to that present in the unabsorbed anti-RMC serum. In other words, previous absorptions had removed most of the unrelated rabbit antibodies from the antiserum and the absorptions enabled a discrete discrimination among mast cell surface antigens having medium mobilities on the electrophorectic gel. In order to establish that sufficient anti-RMC serum had been used to precipitate the mast cell surface antigens, a constant amount of the antiserum was used to precipitate the surface antigens, from different numbers of mast cells. It was found that when the cell number was doubled, the peak height of most radiolabelled surface antigens was also approximately double (Figure 10). This indicates that the antigen was the limiting factor in this particular antigen-antibody system. In other words, there were enough antibodies present to precipitate most of the mast cell surface antigens.

It was, of course, of considerable interest to identify the various peaks and thus the antigens precipitated by anti-RMC_{abs} as seen in Figures 9 and 10 and to establish whether or not the receptor for IgE, or a component thereof, was among these antigens. An inspection of Figure 9 indicated that peak C was a particularly likely candidate for being a component of the receptor for IgE as identified by Conrad and Froese (1976). To check this, the following experiment was performed.

Figure 10. SDS-polyacrylamide gel electrophoretic analysis of RMC surface antigens from different numbers of RMC precipitated by constant amounts of anti-RMC_{abs}.

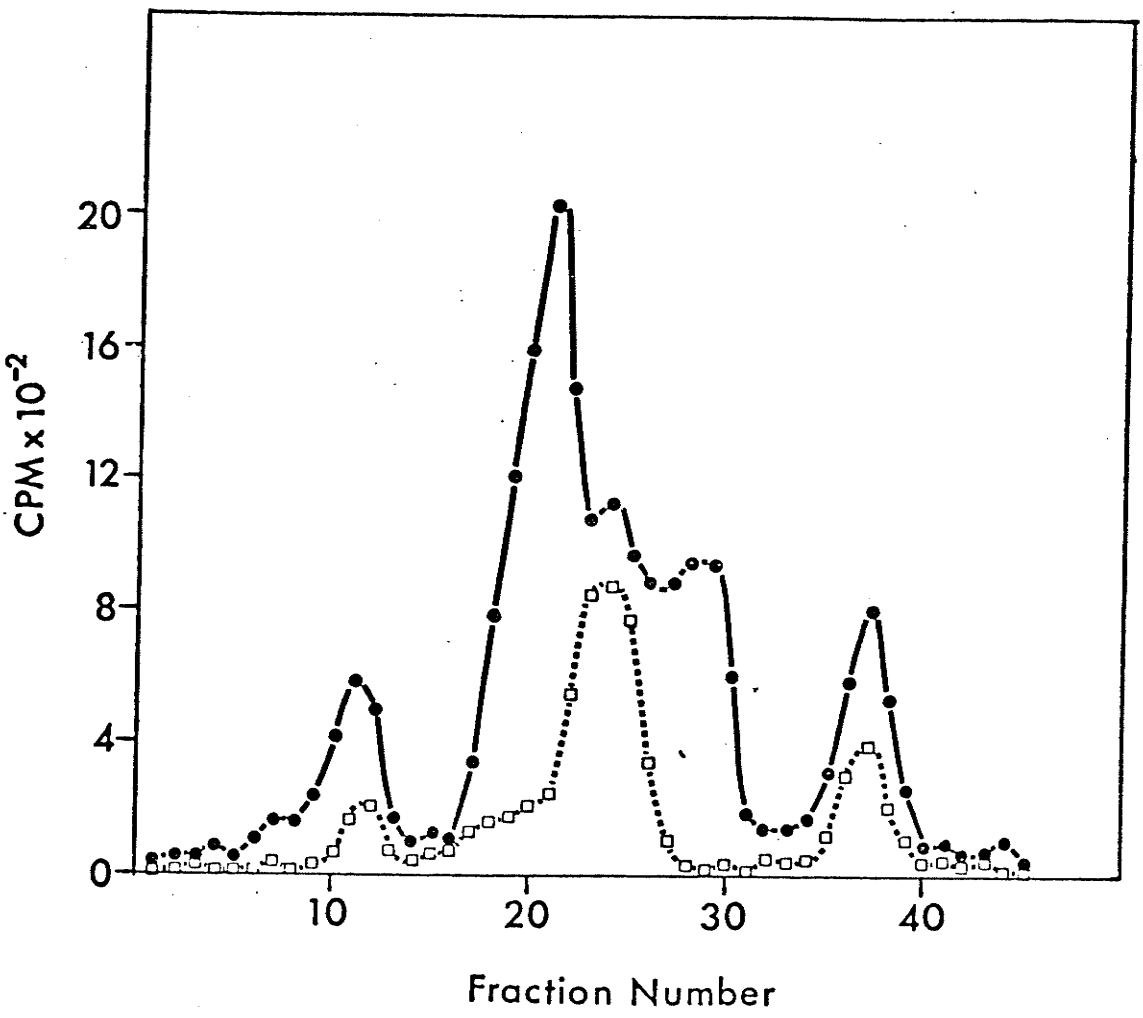
Precipitated surface antigens from $6x10^5$ RMC (□·····□) and from $1.2x10^6$ RMC (●——●).


24
20
16
12
8
4
0
CPM x 10⁻²
10
20
30
40
Fraction Number

One batch of rat mast cells was labelled with ^{125}I, dissolved in NP-40 and the cell surface antigens were precipitated using the anti-RMC_{abs} serum. A second batch was surface labelled with ^{131}I. After washing away the unbound radiolabel, the cells were incubated with 10 µg of IgE and the excess IgE removed by washing. The IgE bound cell surface components were extracted with NP-40 and precipitated with rabbit anti-rat IgE as described by Conrad and Froese (1976), using a sandwich system which consisted of rabbit antiserum specific for rat ε chains and a goat antiserum specific for rabbit immunoglobulins. Both precipitates were solubilized, mixed together and analysed on the same gel by electrophoresis. The various gel fractions were analysed for ^{125}I and ^{131}I activities. The result is shown on Figure 11. Three ^{131}I-labelled bands were revealed by the analysis of the precipitates of RMC which had been incubated with IgE. In addition to a slow and a fast moving bands which had similar electrophorectic mobilities as peaks A and E of Figure 9, respectively, a peak with medium electrophoretic mobility was also present. This particular peak had been successfully characterized by Conrad and Froese (1976), using the same analytical system, as the receptor for IgE or at least a part of the receptor complex. This peak appeared between fractions 22 - 25. Interestingly, a peak with identical mobility was among the antigens precipitated by the anti-RMC_{abs} serum (Figure 11). In fact, this peak is identical to peak C of Figure 9. Thus, while this observation strongly suggests that the receptor for IgE was among the antigens precipitated by the anti-RMC as well as the anti-RMC_{abs} sera, it cannot be taken as an absolute proof that this was really so. Peaks in identical position in a pattern obtained by the SDS polyacrylamide gel

Figure 11. Comparison of RMC surface components precipitated by different antisera.

RMC surface antigens (^{125}I-labelled) precipitated by anti-RMC abs (●——●), are compared to RMC surface components (^{131}I-labelled) precipitated by ε-specific rabbit anti-IgE, after the RMC had been incubated with rat IgE (□·····□).


CPM x 10⁻²
0
4
8
12
16
20
10
20
30
40
Fraction Number

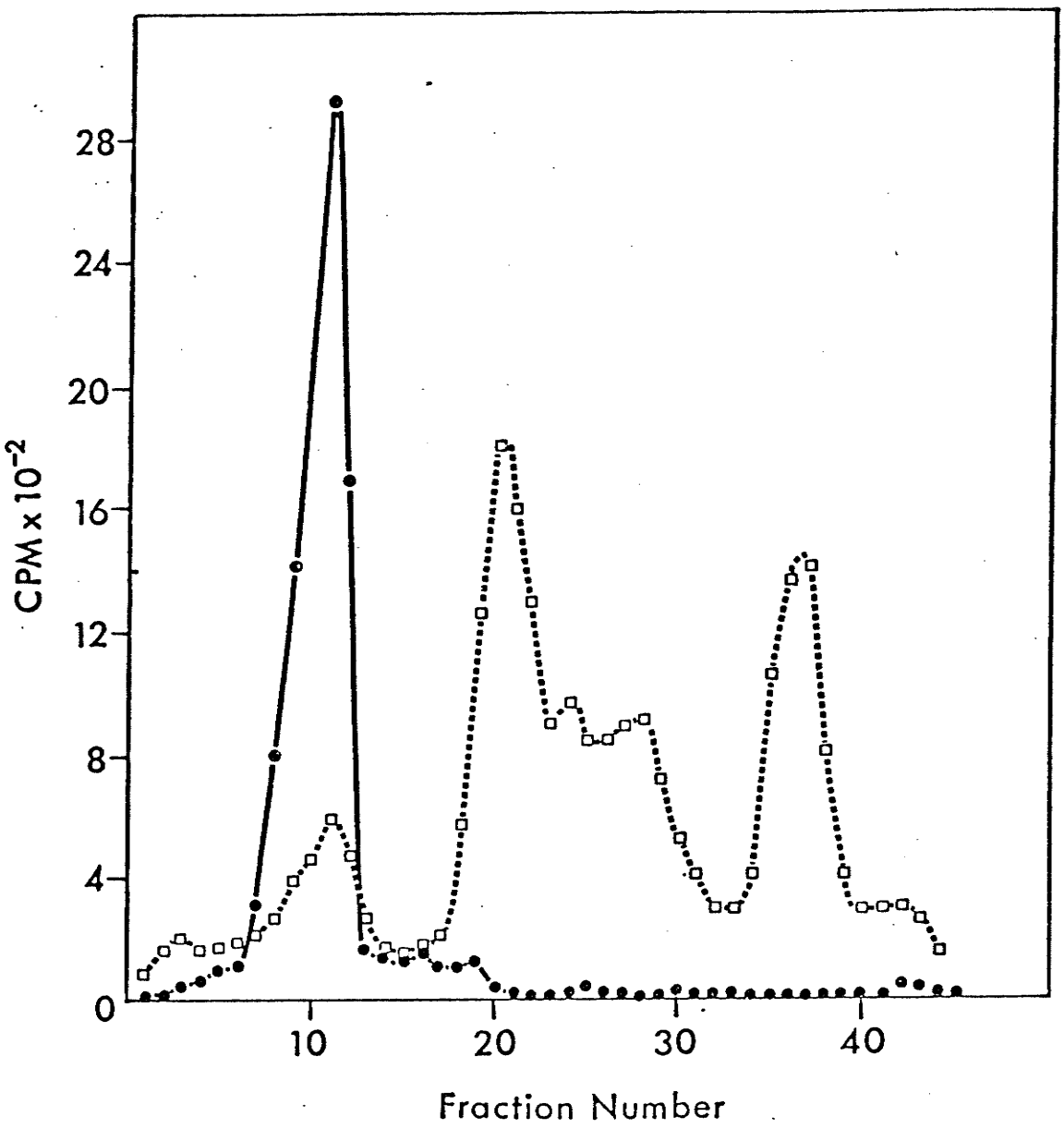
electrophoresis only point to molecules of similar molecular weight but not necessarily of similar biological function.

A peak between fractions 35 - 39 had also been described by Conrad and Froese (1976) and it was considered to be unrelated to IgE or its receptor. It was suggested by these authors that this component might represent a "hetero" Fc receptor, which interacts with the immunoglobulins present in the immune complexes which take part in the precipitation of cell surface components. Such a component could have contributed, at least in part, to the peak seen in this region in both Figures 9 and 11 and precipitated by anti-RMC. It most likely accounts for the small peak revealed by polyacrylamide gel electrophoresis when normal rabbit serum was used for the precipitation (Figure 9).

The peak observed between fractions 10 and 13 (Figure 11) when obtained by precipitation involving anti-IgE and RMC incubated with unlabelled IgE, can be attributed to IgE, present on the surface of RMC at the time of isolation (Conrad and Froese, 1976). Since a component with similar mobility was precipitated by anti-RMC_{abs} (Figure 11, c.f. peak A, Figure 9) further experiments were undertaken in order to demonstrate that IgE has a mobility in this region. When free rat ^{131}I-IgE was used as a marker for the SDS polyacrylamide electrophoresis, a single ^{131}I peak appeared at the same region of the gel where peak A of Figure 9 resides (Figure 12). It is, therefore, conceivable that based on its mobility this high molecular weight surface component precipitated by both anti-RMC and anti-RMC_{abs} represents IgE molecules which were present on the mast cell at the time of isolation. However, in view of the fact that anti-RMC_{abs} had been absorbed with IgE as well as other rat immunoglobulins, it is very unlikely that this peak was indeed

Figure 12. SDS-polyacrylamide gel electrophoretic analysis of RMC surface antigens interacting with anti-RMC_{abs} and of rat IgE.

RMC surface antigens (^{131}I-labelled) precipitated by anti-RMC_{abs} (□•••••□) were analysed together with $5x10^{-3}$ µg of ^{125}I-IgE (●——●) on the same gel.


28
24
20
16
12
8
4
0
CPM x 10⁻²
10
20
30
40
Fraction Number

IgE. For this peak to be IgE anti-RMC_{abs} would have had to contain antibodies capable of interacting with the receptor for IgE when the latter was complexed with IgE. At present, it does seem most likely that this antigen is different from IgE but has a molecular weight similar to that of IgE.

The identitites of peaks B and D as seen in Figure 9 are not clear. However, since both were precipitated by anti-RMC_{abs}, it is plausible to suggest that they probably represent two groups of antigens characteristic of the mast cell surface. It should be noted that the relative intensity of the peaks representing the surface antigens varied from experiment to experiment as can be seen in Figures 9, 10, 11 and 12. This can be explained in several ways. First, the relative number of the various antigens may vary from cell batch to cell batch, or second, there may have been relative differences in the labelling efficiencies of the various antigens. Based on the relative intensity of the various peaks it would appear that the peak which can be seen between fractions 19 - 22 (peak B of Figure 9) represents the major surface antigens or antigens of rat mast cells. However, it must be kept in mind that this would only be the case if equal numbers of ^{125}I molecules had been incorporated into each of the surface antigens. Any antigens having a relatively high percentage of accessible tyrosine residues would have a high specific activity and would thus yield a relatively high peak on polyacrylamide gel electrophoresis. By the same token, any antigen not made up of protein, or not containing any tyrosine residues might not have been detected on the gels. It should be pointed out that each peak observed on the gel electrophorectic pattern could represent an antigen or more than one antigen having similar electrophorectic

mobility and hence similar molecular weight. It is beyond the means of this analytical method to solve this problem. Finally, it should be mentioned that while most of the antigens precipitated by either anti-RMC or anti-RMC_{abs} had been derived from the mast cell surface, the possibility that a few of them were of intracellular origin, cannot be excluded. The lactoperoxidase method of iodination only labels cell surface antigens (Baur et al., 1972). However in view of the fact that viability of mast cells used in these studies varied between 85% and 95%, a few intracellular proteins may have been labelled and consequently precipitated by the antisera.

In conclusion, it should be said that the present study has presented some evidence which suggests fairly strongly that antibodies to the mast cell receptor for IgE are among the antibodies elicited in a rabbit upon immunization with rat peritoneal mast cells in complete Freund's adjuvant. This conclusion is based on the fact that the antiserum, absorbed with liver cells and rat immunoglobulins, including IgE, was still quite effective in inhibiting the binding of IgE to mast cells, and that it precipitated an antigen which, in SDS polyacrylamide gel electrophoresis, had a mobility identical to that of a molecule identified by Conrad and Froese (1976) to be the receptor for IgE or a component thereof. As mentioned above, the results presented here only suggest that the antiserum contains antibodies to the receptor although they cannot prove it beyond any doubt.

SUMMARY

Several rabbits were immunized with purified rat peritoneal mast cells (RMC) in complete Freund's adjuvant and resultant antisera were collected. The serum with the most pronounced anti-RMC activity was selected and absorbed with rat immunoglobulins, including IgE, and liver cells in order to render it specific for RMC. This absorbed anti-RMC serum was no longer cytotoxic to rat lymph node cells but remained so to RMC. It was capable of inducing a skin reaction in rats similar to a reverse PCA reaction, but it did not react with rat immunoglobulins including IgE. This serum also inhibited the binding of ^{125}I-IgE to normal rat mast cells. A rabbit anti-rat sarcoma serum, absorbed with rat liver cells and used as a control, could not be shown to interact with RMC. In order to identify the cell surface antigens of RMC interacting with the absorbed anti-RMC serum, RMC was surface labelled with ^{125}I, and solubilized in Nonidet P-40. Surface antigens were then precipitated using a sandwich system consisting of the absorbed anti-RMC serum and a goat anti-rabbit immunoglobulin serum. When the precipitates were dissolved in sodium dodecyl sulfate (SDS) and analyzed by SDS-polyacrylamide gel electrophoresis, five clearly distinguishable radioactive peaks were observed. One of these had a mobility and thus a molecular weight similar to the receptor for IgE. These results suggest, even though they do not provide a definite proof, that antibodies to the receptor for IgE were present in the anti-RMC serum.

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