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Transplantable Immunoglobulin-secreting Tumours in Rats

IV. SIXTY-THREE IgE-SECRETING IMMUNOCYTOMA TUMOURS

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Summary. The inbred LOU/Wsl rat strain presents a high incidence of spontaneous ileocoecal immunocytoma which secrete monoclonal immunoglobulins. These tumours are transplantable in histocompatible animals and their secreting properties are maintained over many passages.

A screening of monoclonal immunoglobulins secreted by 250 such tumours revealed sixty-three proteins which shared class-specific determinants, physicochemical features and biological properties different from those of rat IgM, IgA, IgG1, IgG2a, IgG2b and IgG2c classes, and characteristic of proteins of the IgE class.

INTRODUCTION

Reaginic antibodies have been shown to exist in the rat (Mota, 1963; Binaghi and Benacerraf, 1964; Becker and Austen, 1966; Austen, Bloch, Baker and Arnason, 1965) and to have properties similar to those of man (Binaghi and Benacerraf, 1964; Binaghi, Benacerraf, Bloch and Kourilsky, 1964; Jones and Ogilvie, 1967; Bloch and Wilson, 1968). Recently two different papers have described what appears to be the rat equivalent of IgE (Stechschulte, Orange and Austen, 1970; Jones and Edwards, 1971).

In all species thus far studied, IgE appears to exist only in trace amounts. The study of the physicochemical properties of this class of immunoglobulins therefore largely depends on the dicovery of tumours that produce massive quantities of IgE. However, only three cases of IgE myeloma have been reported (Johansson and Bennich, 1967; Ogawa, Kochwa, Smith, Ishizaka and McIntyre, 1969; Fishkin, Orloff, Scaduto, Borucki and Spiegelberg, 1972) in man, and only two of these have furnished sufficient material for structural studies on IgE. To our knowledge, no IgE-producing tumour has been reported in animals.

The inbred LOU/Wsl rats present an astonishingly high incidence (23 per cent) of spontaneous immunocytoma tumours arising from the ileocoecal lymph node, between the age of 8 months and the end of their life (Bazin, Deckers, Beckers and Heremans, 1972; Bazin, Beckers, Deckers and Moriamé, 1973a).

The peak incidence is situated at 12 months. Seventy-four per cent of these immunocytomas synthesize monoclonal immunoglobulins, including Bence-Jones proteins. Among the myeloma proteins, 34 per cent are of a class identified in this paper as being IgE. This represents an over-all incidence of about 8 per cent. Sixty-three of such IgE-secreting rat immunocytomas have been observed to this date.

MATERIALS AND METHODS

Rats

The rats developing the immunocytomas were of the LOU/C/Wsl and LOU/M/Wsl inbred strains and have been described previously (Bazin *et al.*, 1972; Bazin *et al.*, 1973a). Wistar R and Sprague–Dawley rats were also employed for rosette or passive cutaneous anaphylaxis (PCA) tests.

Immunocytomas

The gross microscopical and cytological anatomy, transplantation and maintenance *in vivo* of the LOU immunocytomas have been reported elsewhere (Bazin *et al.*, 1972). The serum levels of the IgE monoclonal proteins in these rats are quite variable and may reach as high as 50–60 mg/ml in some cases (Bazin, Beckers and Querinjean, 1973b). These tumours can be propagated by transplantation in ascitic or solid form in syngeneic hosts in which they generally grow quite fast. The tumours retain their capacity for immunoglobulin secretion during successive transplantations, although dedifferentiation may occur, particularly when the IgE immunocytomas are transplanted in ascitic form.

Preparation of IgE monoclonal proteins

The serum or ascitic fluid collected from tumour-producing rats was diluted twice with saline and precipitated by ammonium sulphate at 40 per cent final concentration. The precipitate was washed with 45 per cent saturated ammonium sulphate, resuspended in saline and dialysed against 0·05 M Tris–HCl buffer, pH 8·1. This fraction was applied to a DEAE–cellulose (DE-32, Whatman) column and eluted with a convex gradient controlled by an Ultrograd (LKB) apparatus. The starting buffer was 0·05 M Tris–HCl, pH 8·1, and limit buffer 0·10 M Tris, 0·40 M NaCl, pH 8·1.

The fractions containing immunoglobulin E were pooled, concentrated by ultrafiltration, dialysed against 0·2 M Tris, 0·15 M NaCl, 1 per cent sodium azide, pH 8·0. The immunoglobulins were purified by two successive gel-filtrations on Sephadex G-200 (5 × 90 cm columns).

Preparation of antisera

Antisera against IR2 or IR16 monoclonal immunoglobulins of the IgE class were obtained by injecting the purified proteins into goats or rabbits. Antisera were absorbed with a solid immunosorbent (Avrameas and Ternynck, 1969) made of serum from germ-free rats. The preparation of antisera specific for rat IgM, IgA, IgG1, IgG2a, IgG2b, IgG2c and kappa light chains, is described elsewhere (Bazin *et al.*, 1973b).

Immunodiffusion analysis

Ouchterlony analyses, immunoelectrophoresis and agarose electrophoresis techniques have been described (Querinjean, Bazin, Beckers, Deckers and Heremans, 1972).

Acrylamide gel electrophoresis

The electrophoresis was carried out in gel slabs of 7 per cent acrylamide with the gel buffer consisting of 0·075 M Na_2HPO_4, 0·025 M NaH_2PO_4, pH 7·0, containing 1 per cent sodium dodecylsulphate and 5 M urea. The electrophoresis was run for 3 hours at 100 V

and 100 mA, in 15 cm gels, with a similar running buffer without urea. The samples (7 μg) were dissolved in the gel buffer containing 8 M urea. For certain experiments the samples were reduced with 2 μl 2-mercaptoethanol for 20 μl of sample solution, and heated, for 15 min at 60°C.

Ultracentrifugation

The sedimentation velocity analysis was performed with a Spinco Model E in Kel-F cells at 52,800 rpm in M/15 Sørensen buffer, pH 7·4. The proteins were dissolved in this buffer and analysed at four different concentrations ranging from 4 to 14 mg/ml.

Rosette technique

Peritoneal cells collected from normal Wistar R rats after intraperitoneal injection of Tyrode solution as modified by Bloom, Fredholm and Haegermark (1967), were incubated with rabbit antisera to IgE, IgG1 or IgG2 rat immunoglobulins employed in great excess. After twenty minutes at 37°, the cells were washed three times with the Tyrode solution. They were then incubated with sheep red blood cells coated with monoclonal rat immunoglobulins of different classes (IgE, IgG1 or a mixture of IgG2a, IgG2b and IgG2c) following techniques described by Herbert (1967); use being made of bis-diazotized benzidine as the coupling agent. The presence or absence of rosettes was verified after short centrifugation and resuspension in Tyrode solution. Mast cells were stained by Neutral Red as indicated by Wolfromm (1972).

Passive cutaneous anaphylaxis (PCA)

The method described by Goose and Blair (1969) was used with slight modifications. Reagin-rich serum directed against *Nippostrongylus brasiliensis* was obtained 35 days after experimental infestation of Sprague–Dawley rats. The PCA titre of the pool was 1/128. Its IgE concentration was about 16 μg/ml. PCA activity of this serum was completely abolished by heating at 56°C for 4 hours.

PCA tests were carried out by injecting an extract of adult *N. brasiliensis* worms intravenously 72 hours after sensitization of the skin. The following scoring system was used to describe the reactions, as read on the inner skin surface. 0 = diameter < 5 mm. 1 = diameter from 5 to 10 mm. 2 = diameter from 10 to 15 mm. 3 = diameter from 15 to 20 mm. 4 = diameter > 20 mm. The mean value of the scores was computed in each experiment.

Reverse cutaneous anaphylaxis (RCA)

The animals were injected with Evans Blue dye (25 mg/kg) by the intravenous route. Within 5 minutes, 0·1 ml of the IgG fraction from antiserum specific for one of the seven rat immunoglobulin classes was injected intradermally and the reactions were read 5 minutes later, as described above. The different antisera used were fractionated on a DEAE-cellulose column equilibrated with a Tris–HCl buffer, pH 8.0. Their IgG fractions eluted at 0·05 M retained their precipitation properties as verified by Ouchterlony analysis.

*Absorption of rat anti-*Nippostrongylus *serum by specific anti-immunoglobulin sera*

Increasing amounts (0–35 μl) of rabbit antisera specific for the various rat immunoglobulin classes were added to 0·5 ml of rat anti-*Nippostrongylus* serum. The mixtures were kept overnight at 4°C. After centrifugation (27,000 ***g***), each supernatant was used to

sensitize two rats. One side of each rat was used for the assay, the other side serving as a control, for tests with normal rabbit serum instead of the specific anti-immunoglobulin reagents.

*Competition between IgE monoclonal protein and rat anti-*Nippostrongylus *antibodies*

A mixture of 0·5 ml of rat anti-*Nippostrongylus* serum and 0·05 ml of IR2 protein (= IgE) solution (2·7 mg/ml) was prepared. As a control, the same rat serum was diluted similarly with saline.

A PCA test was performed on four rats, the serum + IR2 mixture being injected in one side, and the control mixture in the other side.

RESULTS

IMMUNOCHEMICAL CHARACTERIZATION OF A CLASS OF MONOCLONAL RAT PROTEINS DIFFERENT FROM IgG, IgA OR IgM

The immunoglobulin nature of the IR2 monoclonal rat protein was attested by its possessing κ-chain antigenic determinants, as shown by an Ouchterlony test with anti-κ serum, using S210 rat Bence-Jones protein as the reference antigen (Fig. 1). Protein

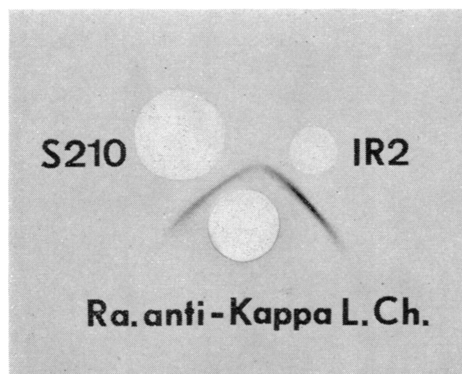

FIG. 1. Demonstration of L chain determinants in the IR2 protein. S210 = purified kappa light chain (0·1 mg/ml). IR2 = purified monoclonal protein (4 mg/ml). Central well = rabbit antiserum specific for rat kappa chains.

IR2 did not precipitate with antisera specific for rat IgM and IgA (not shown); and it did not cross react with IgG1, IgG2a, IgG2b and IgG2c, as indicated by comparative gel diffusion precipitin reactions (Fig. 2) produced by testing IR2 rat serum with antisera against these four different rat IgG sub-classes and against an antiserum raised against IR 162 monoclonal rat immunoglobulin (referred to as anti-IgE in the figure).

Sixty-two further rat immunocytoma proteins were found to give precipitin reactions identical to those of protein IR2.

INHIBITION OF PCA ACTIVITY OF RAT ANTI-*Nippostrongylus* SERUM ABSORBED WITH ANTISERUM TO PROTEIN IR2

The undiluted anti-*Nippostrongylus* serum (0·5 ml) was found to have lost part of its PCA activity after absorption with 15 μl of anti-IR2 serum and was completely inacti-

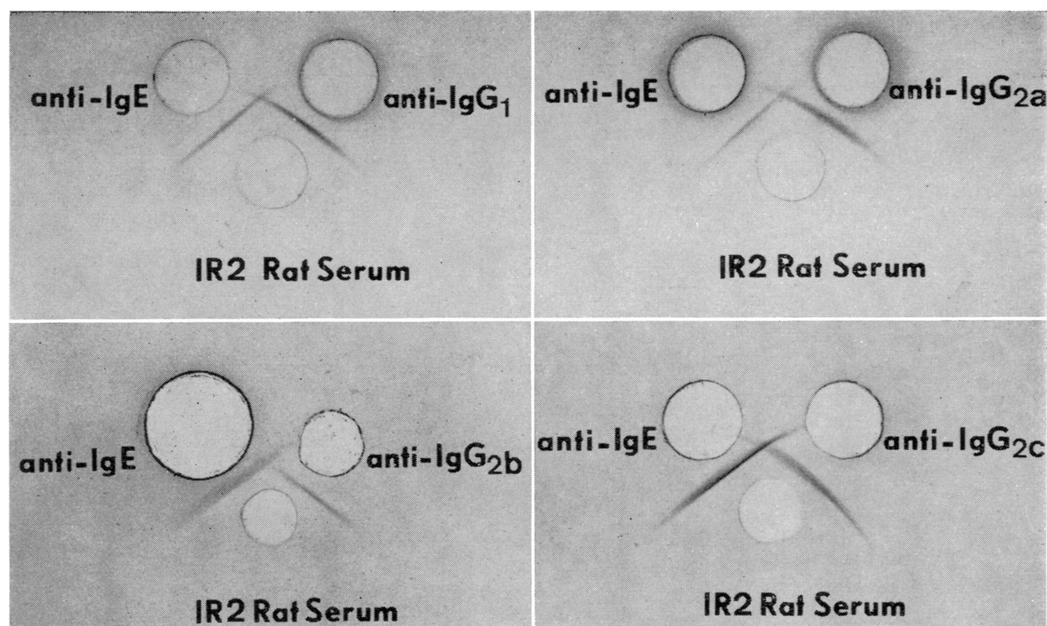

FIG. 2. Absence of cross-reactivity of the IR2 protein and four different IgG rat immunoglobulins. Anti-IgE = rabbit antiserum against IR 162 protein (IgE class). Anti-IgG1, anti-IgG2a, anti-IgG2b and anti-IgG2c = rabbit antisera against the four different rat IgG heavy chain subclasses. IR2 rat serum = serum of IR2 tumour-carrying animals at appropriate dilutions.

vated by absorption with 35 μl of this serum (Table 1). The absorption with the other anti-immunoglobulin sera (IgM, IgA, IgG1, IgG2a, IgG2b, IgG2c) did not decrease the intensity of the PCA reaction.

COMPETITION BETWEEN THE IR2 PROTEIN AND ANTI-*Nippostrongylus* ANTIBODIES FOR BINDING SITES IN THE SKIN

To prove the affinity of IR2 protein for rat skin tissues, four rats were sensitized with different mixtures of IR2 protein and anti-*Nippostrongylus* serum.

A clearcut inhibition was observed with 0·32 mg of IR2 protein per 1 ml of anti-*Nippostrongylus* serum, the score of the PCA reaction being 1·25 for this mixture against 3·5 for the control with saline instead of IR2 protein.

REVERSE CUTANEOUS ANAPHYLAXIS (RCA)

Of all the antisera to the different rat immunoglobulin classes tested, only those directed against IR2 immunocytoma protein gave a very strong reaction when injected into the skin of normal unsensitized rats (Table 2).

AFFINITY OF THE IR2 PROTEIN FOR MAST CELLS

Washed mast cells from Wistar R rats incubated with antiserum to IR2 monoclonal protein were found to fix anti-IR2 antibodies, as attested by their capacity to form rosettes

TABLE 1

PCA SCORES OBSERVED AFTER ABSORTPION OF RAT ANTI-*Nippostrongylus* SERUM BY ANTI-IR2 SERUM

Volume added (μl)	Incubation with anti-IR2 serum	Incubation with saline
0	4 and 4	4 and 4
5	4 and 4	4 and 4
10	4 and 4	4 and 4
15	3 and 3	4 and 4
20	3 and 3	4 and 4
25	2 and 2	4 and 4
30	1 and 1	4 and 4
35	0 and 0·5	4 and 4

Half a millilitre of anti-*Nippostrongylus* serum was incubated overnight with various increasing amounts of anti-IR2 serum or saline. Each incubation mixture was used to sensitize two rats and the passive cutaneous anaphylaxis reactions were performed 3 days later.

TABLE 2

SCORES OF THE RCA TESTS PERFORMED WITH ANTISERA SPECIFIC FOR THE DIFFERENT RAT IMMUNOGLOBULIN CLASSES

Reagents	RCA scores			
B7 (anti-IR2 serum)	3	4	4	4
L606 (anti-IR2 serum)	4	4	—	—
L669 (anti-IgA serum)	1·5	1	—	—
L 626 (anti-IgG1 serum)	1	1	—	—
L59 (anti-IgG2a serum)	0·5	1	—	—
L471 (anti-IgG2b serum)	1	1	—	—
L680 (anti-IgG2c serum)	1	0·5	—	—
L515 (anti-IgM serum)	0·5	1	—	—
Normal rabbit serum	2	1·5	1	0·5
Saline	1	0·5	0·5	1

Reverse cutaneous anaphylaxis was induced by the injection of 0·1 ml of the IgG fraction of the different antisera or saline into the skin of normal unsensitized rats previously injected with Evans Blue dye.

with IR2-coated sheep red cells. Controls with antisera to rat IgG1 or IgG2 and IR2-coated erythrocytes, or with anti-IR2 serum and erythrocytes coated with rat IgG1 or IgG2 were negative.

ELECTROPHORETIC MOBILITY

In agarose gel, at pH 8·6 (Fig. 3), the IR2 protein as well as six further monoclonal rat proteins belonging to the same antigenic class (IR 104, 152, 159, 162, 183 and 192) were found to migrate as relatively homogenous components, although their bands were not quite as sharp as those of IgG class proteins such as the IR 27 (IgG1), included in the figure. All were situated in the β_2 range, except one (IR 192) which migrated as a slow γ-globulin.

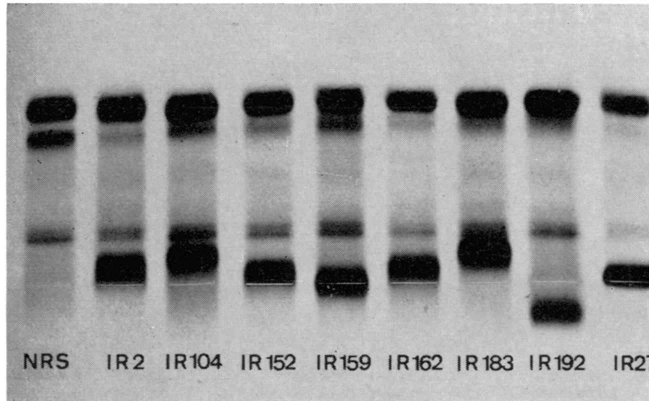

FIG. 3. Agarose electrophoresis of serum from rats carrying different tumours (IR 104, IR 152, IR 159, IR 162, IR 183, IR 192) secreting IR2-like (IgE) proteins. Normal rat serum (NRS) and serum from a rat with an IgG1 immunocytoma (IR27) are shown for comparison.

PRECIPITATION REACTIONS OF ANTISERUM TO THE IR2 PROTEIN WITH NORMAL AND HEATED ANTIGENS

A very faint precipitate was produced when rabbit or goat antisera to the IR2 protein were mixed with the serum of normal rats from every strain tested. A corresponding weak precipitin line was also obtained in Ouchterlony plates and was found to be identical to IR2 protein (Fig. 4). These reactions were completely abolished when the rat serum was heated at 56°C for 4 hours. Immunocytoma proteins of the IR2 type were likewise found to become non-precipitable by their antisera upon heating.

DEAE–CELLULOSE CHROMATOGRAPHY OF PROTEINS OF THE IR2 CLASS

Fig. 5 shows the elution diagram of serum from LOU/Wsl rat with the IR2 immunocytoma after precipitation by 40 per cent ammonium sulphate. The elution pattern of

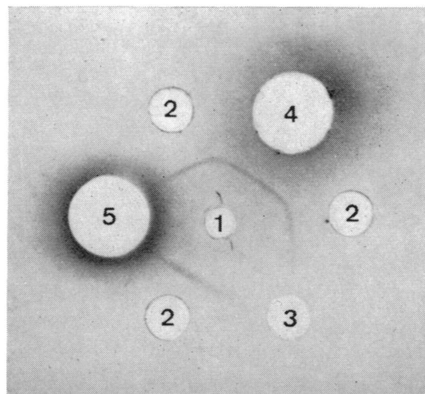

FIG. 4. Heat lability of normal rat serum IgE and IR2 monoclonal protein. (1) Rabbit antiserum to the IR2 protein. (2) Purified IR2 protein (0·1 mg/ml). (3) Purified IR2 protein (0·1 mg/ml) heated at 56° during 4 hr. (4) Normal rat serum. (5) Normal rat serum heated at 56° during 4 hr.

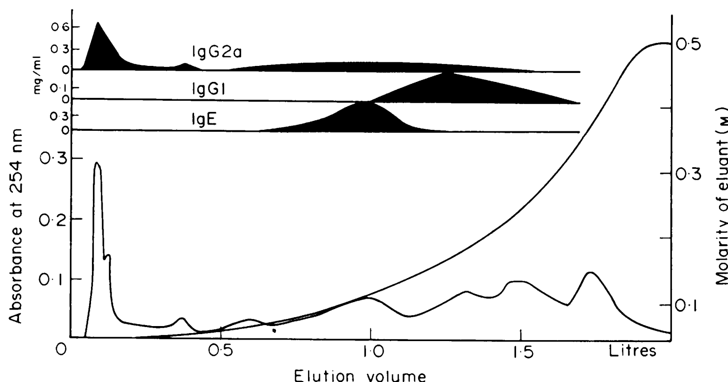

FIG. 5. DEAE-cellulose chromatography of 40% ammonium sulphate precipitate of 27 ml serum from a rat with the IR2 (= IgE) tumour. Upper part = concentrations of IgG1, IgG2a and IgE as detected by Mancini tests performed on alternate fractions.

IR2 protein, as compared to normal IgG1 and IgG2a rat immunoglobulin was revealed by immunodiffusion test using specific antisera.

GEL-FILTRATION ON SEPHADEX G-200

On Sephadex G-200, the IR2-like protein from IR88 immunocytoma serum was eluted with the first part of the second peak (see Fig. 6), indicating a molecular size intermediate between that of the IgM and IgG markers.

On a calibrated column (2·5 x 90 cm) of Sephadex G-200 the Ve/Vo ratios for rat IgG were found to obey the relationship of Whitaker (1963). On this column, four different preparations of the IR2-like IR 162 rat protein furnished a molecular weight estimate of 198,000 (±10,000) Daltons of rat IgE IR 162 myeloma protein.

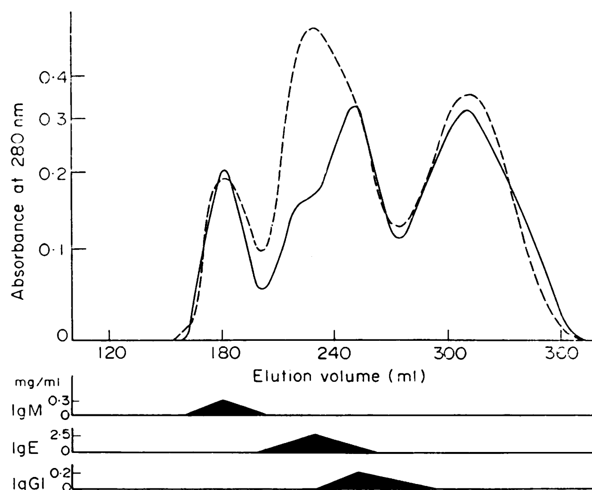

FIG. 6. Gel-filtration on Sephadex G-200 (2·5 x 90 cm column) of (—) 3 ml of fresh normal rat serum and (- - -) 3 ml of fresh serum from a rat with the IR 88 (IgE) tumour. Lower part = concentrations of IgM, IgE, IgG1 as detected by Mancini on the different fractions.

SDS–ACRYLAMIDE GEL ELECTROPHORESIS

Unreduced samples of IR2-like rat immunoglobulins exhibited an electrophoretic mobility in 5 per cent acrylamide gels that was definitely slower than that of different monoclonal IgG proteins.

After reduction, the light chains of IR2-like proteins migrated in 7 per cent acrylamide gels like those of IgM, IgA, IgG2a and IgG2c.

By the same technique, and with trypsin (mol. wt = 23,000), rat immunoglobulin light chains (mol. wt = 23,500), ovalbumin (mol. wt = 45,000), bovine serum albumin (mol. wt = 67,000), human transferrin (mol. wt = 76,000), and bovine serum albumin dimer (mol. wt = 134,000) as markers, the molecular size of the heavy chains of different classes of monoclonal rat immunoglobulins was evaluated (Table 3).

From these data, the mol. wt of the IR2-like proteins would appear to be 183,000.

TABLE 3

MOLECULAR WEIGHT OF RAT HEAVY CHAINS IN SDS–7 PER CENT ACRYLAMIDE GELS

IgA (IR22)	58,000
IgE (IR162)	68,000
IgG1 (IR27)	55,000
IgG2a (IR25)	54,000
IgG2c (IR64)	54,000

ULTRACENTRIFUGATION

The purified IR2 protein sedimented as a fairly homogenous peak in the ultracentrifuge (Fig. 7), and furnished an $S^{2}_{00,w}$ value of 7·6S, as compared to 6·4S and 6·8S for IgG2a (IR33) and IgG1 (IR27) immunoglobulins respectively analysed under the same conditions.

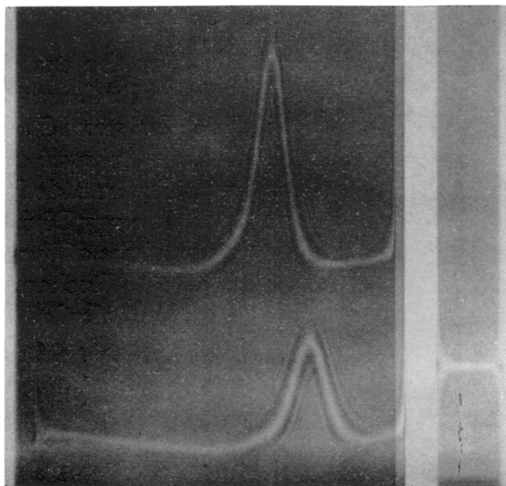

FIG. 7. Ultracentrifugation pattern of purified IR2 (= IgE) protein. Upper part = 12 mg/ml. Lower part = 7·3 mg/ml. Sedimentation from left to right. (52,800 rev/min.)

DISCUSSION

Among the 250 ileocoecal LOU/Wsl rat immunocytomas examined, sixty-three were found to secrete an immunoglobulin, which had antigenic and physico-chemical properties different from rat IgM, IgA and the four different classes of IgG described elsewhere (Bazin *et al.*, 1973b).

The most characteristic physico-chemical property of these monoclonal proteins was a molecular size larger than that of rat IgG. This feature was demonstrated on the intact protein by gel filtration, SDS–acrylamide gel electrophoresis of unreduced samples, and ultracentrifugation. The characteristic molecular weight was due to the heavy chain as observed by SDS–acrylamide gel electrophoresis of reduced IR2-like proteins.

The class-specific determinants of these proteins resulted in the production of an antiserum which precipitated with all IR2-like monoclonal proteins, but not with normal or monoclonal IgM, IgA or IgG rat immunoglobulins. By means of this antiserum it was possible to demonstrate that IR2-like proteins existed at very low concentration (about 16 μg/ml) in normal rat serum.

All these properties are very similar to those described for the peculiar IgE-like rat immunoglobulin class which has been described as the carrier of reaginic activity (Ogilvie, 1967; Jarrett and Steward, 1973). The validity of this assumption was demonstrated by showing that antiserum specific for IR2-like monoclonal proteins abolished the PCA activity of a rat serum containing reaginic antibodies to *Nippostrongylus brasiliensis*. The IR2 protein was also shown to compete with these reaginic antibodies in the PCA test.

Furthermore the antiserum to IR2-like proteins reacted with natural reaginic IgE antibodies fixed on the mast cells in the skin of normal rats, as demonstrated by reverse cutaneous anaphylaxis as well as by the rosette experiments.

From this combined physico-chemical and biological evidence, it was concluded that the sixty-three IR2-like monoclonal immunoglobulins described here were representative of the rat IgE class.

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