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Study of the Antigenic Constituents of Sera from Mouse/Rat Chimaeras

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Summary. The sera of CBA mice lethally irradiated and restored with rat bone marrow or foetal liver have been examined by immunoelectrophoresis at various times after irradiation. Such sera contain several specific rat proteins including γ globulin, at least two α_2 globulins, one or two β_1 globulins and one β_2 globulin. A large number of specific mouse proteins are also present, but no mouse γ globulins have been detected.

The rat constituents appear progressively and persist for long periods; this implies that they are synthesized by rat cells.

There is a close correlation between the presence of rat γ globulin in the serum and the presence of dividing rat cells in the bone marrow and spleen.

The fact that γ globulin is solely of rat specificity does not necessarily rule out the possibility of immune activity by the host against the graft.

Several of the normal serum proteins, in addition to the γ globulins, originate from the injected cells, and are therefore presumably not synthesized by the liver.

In chimaeras grafted with rat skin, the number of specific rat serum proteins is often larger than the number in chimaeras which have not been grafted.

INTRODUCTION

In a short preliminary publication (Grabar, Courcon, Ilbery, Loutit and Merrill, 1957) we described the results of immuno-chemical analysis of the sera of lethally X-irradiated mice injected with suspensions of rat bone-marrow cells. These analyses showed that the serum of such animals contains simultaneously proteins of both species — particularly rat γ globulin and other proteins (e.g. albumin) of mouse specificity. Similar results were obtained by Weyzen and Vos (1957). By contrast, Gengozian (1959) criticized our observations on the basis of results obtained by means of different and less precise methods than ours.

Since our first publication we have performed experiments on numerous animals and under various conditions. The results which we have obtained confirm our previous conclusions and provide also supplementary information.

MATERIALS AND METHODS

IMMUNE SERA. Several series of adult rabbits were immunized with serum from mouse or rat. After a preliminary examination of each serum the best were mixed so as to obtain sufficiently large pools. Two pools of anti-mouse and two pools of anti-rat serum were used

The first pair of pools came from rabbits which had received at a week's interval two intramuscular injections of the antigens with Freund's adjuvant (the first containing 0.12 ml. serum and the second 0.25 ml.), and then during the next 3 weeks four times weekly intravenous injections of the serum diluted 1 in 8 (the first week 0.5 ml., the second 1 ml. and the third 2 ml.). This immune serum was usable once the antibodies which gave cross-reactions had been absorbed, but no longer gave precipitation with certain of the antigens.

We therefore modified the scheme of immunizing the rabbits and prepared a second pair of pools of immune serum; each rabbit received intramuscular injections twice weekly of the following doses of rat or mouse serum: 0.1; 0.15; 0.2; 0.25; 0.3; 0.35 and 0.4 ml.

The rabbits were bled 8 to 10 days after the last injection of antigen. Almost always we used for analyses not the immune sera as such, but solutions enriched in antibodies. To achieve this, we precipitated the sera at one-third saturation of ammonium sulphate; the precipitated fraction after dialysis was finally dissolved in a portion of the initial serum equal to one-tenth of the volume used for preparing the fraction. By this means it was possible to increase the concentration of antibody $2\frac{1}{2}$ to 3 times.

The immune sera and the solutions of antibody thus prepared were absorbed in such a manner as to render them absolutely specific 'anti-rat' and 'anti-mouse'. The absorption was controlled by ring tests and immuno-electrophoretic analyses with the corresponding sera from normal animals of the same strain. Generally the cross reactions were initially very strong and the absorption considerably diminished the titre of antibodies in the anti-mouse immune sera absorbed with rat serum. Nevertheless after complete absorption the immune sera still contained enough antibodies to enable one to distinguish at least ten constituents of normal mouse serum. The anti-rat immune sera on the other hand, even after absorption with normal mouse serum, retained a large proportion of all the antibodies. The first pair of pools was used for Experiments A4 and C, and the second pair for the remainder.

ANIMAL STOCKS. CBA/H mice aged 4-5 months were used throughout these experiments. Nearly all were males. The rats were inbred albino animals of the Harwell stock.

IRRADIATION OF ANIMALS. Mice were irradiated from below in their aluminium living-cages as described by Corp (1957). A dose of 1000 rads total-body X-irradiation (250 kV; 14 mA; H.V.L. 1.2 mm. Cu) was used except when otherwise specified. This is 98–100 per cent lethal to our CBA mice within 14 days.

PREPARATION OF RAT BONE-MARROW CELLS. The ends of femora, tibiae and ilia were avulsed with bone forceps. The marrow from the tubular diaphysis was blown out by compressed air into Tyrode's solution. A cell suspension was prepared by flushing the marrow so obtained several times through a hypodermic needle, and was diluted with further Tyrode's solution to the required concentration. 0.4 ml. of suspension containing $1–2 \times 10^7$ cells, was injected intravenously into each mouse.

Preparations of 'killed' rat bone marrow were obtained in a similar manner. 3.2×10^8 cells were suspended in 2 ml. of Tyrode's solution and irradiated with 10,000 rads. Two Siemens constant potential machines, each operating at 15 mA, 250 kV, crossfired to give X-rays with a half value layer of 1.2 mm. Cu at a total dose-rate of 1250 rads/minute.

PREPARATION OF BONE MARROW EXTRACT. Bone-marrow cells ($>10^8$) were obtained as described above and suspended in 0.5 ml. Tyrode's solution. The suspension, chilled in an iced-water bath, was subjected by means of an MSE-Mullard Ultrasonic Disintegrator to vibration at a frequency of 20 kilocycles/second for 1 minute. On one occasion the prep-

aration was allowed to settle overnight at 0–4° before removal of the supernatant for use. On a second occasion the preparation was centrifuged at 2000 *g* for 10 minutes in a chilled centrifuge.

PREPARATION OF EMBRYO RAT LIVER CELLS. A female rat was killed at approximately 15 days of pregnancy. The embryos were removed and portions of liver were washed in sterile Tyrode's solution. After cell counting, the concentration was adjusted to provide 1.5×10^7 rat cells in 0.4 ml. for intravenous injection into each of 10 irradiated CBA mice.

GRAFTING OF SKIN, THYMUS AND SPLEEN. Full thickness skin grafts approximately 1 cm. in diameter were applied by essentially the method of Billingham and Medawar (1951).

Thymus and spleen were chopped into pieces of about 1 cu. mm. Thymus was inserted subcutaneously in the axillary region. Spleen was inserted intraperitoneally. In each case six to eight pieces were used for each animal.

COLLECTION AND ANALYSIS OF SERA. Mice were bled from the tail or by cardiac puncture at intervals after treatment ranging from 15 minutes to several months. Cardiac puncture was performed under anaesthetic, and the animals were then killed.

The serum of individual animals was analysed by the method of immunoelectrophoresis as described previously (Grabar and Williams, 1955; Grabar, 1958). In the majority of cases, the small amount of serum available, combined with our desire to make all the analyses in duplicate, obliged us to use plates of 4.4×10.7 cm. or even the micromethod of Scheidegger (1955), that is to say gels made on microscope slides. The method of double diffusion in agar was also used (Ouchterlony, 1949).

Some of the analyses were made on fresh serum, while others were made on samples of serum preserved by freezing for various lengths of time or frozen samples transported from Harwell to Paris in vacuum flasks. No notable differences have emerged in the results from using samples preserved in this way.

CYTOLOGICAL EXAMINATION OF DIVIDING CELLS. Mitotic cells in the bone marrow, spleen and lymph nodes of killed animals were examined in metaphase by the technique of Ford and Hamerton (1956). Rat and mouse cells could readily be distinguished (Ford, Hamerton, Barnes and Loutit, 1956). The figures quoted in this paper are based on the pooled score for bone marrow and spleen. Few cells were counted in preparations of lymph node, but, in general, the ratio of mouse to rat cells was similar in all three tissues of any individual.

RESULTS

Three series of experiments were performed. In the first and largest (Series A) mice received lethal X-irradiation followed by an intravenous injection of rat bone marrow. In the second, mice injected with embryonic rat liver were studied in parallel with animals given bone marrow. In the third, mice were injected with bone marrow and subsequently grafted orthotopically with rat skin, subcutaneously with rat thymus or intraperitoneally with rat spleen. It seemed possible that extra proteins of rat specificity might be detectable in animals so grafted.

EXPERIMENT A1

Ten CBA♂ mice were irradiated with 1000 rads and, within 2 hours, injected intravenously with 1.8×10^7 rat♂ bone-marrow cells. Ten non-irradiated CBA♂ mice were injected with 1.1×10^7 rat♂ bone-marrow cells and served as controls.

The immunoelectrophoretic analysis of mouse chimaera sera, taken a sufficient time after the injection of marrow, enables one to distinguish constituents of rat specificity, which form arcs of precipitation with anti-rat serum completely absorbed with normal CBA mouse serum. A typical immunoelectrophoretic analysis of serum taken 44 days after induction of chimaerism is seen in Fig. 1. As in our previous experiments one can almost always observe several precipitating arcs; this proves the existence of several

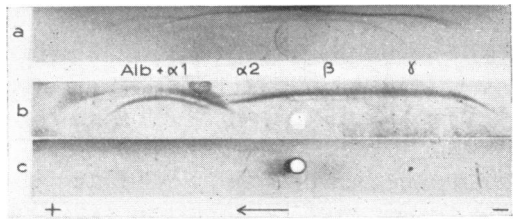

FIG. 1. Immunoelectrophoretic analysis of sera using rabbit anti-rat serum absorbed with normal mouse serum:
(*a*) Serum of mouse/rat chimaera bled 42 days after irradiation;
(*b*) Normal rat serum;
(*c*) Normal mouse serum.

specific rat constituents possessing various mobilities. Fig. 2 is a schematic representation of the results of analysis of four different sera from chimaeras bled 42 days after irradiation. Each serum was analysed both with an anti-rat immune serum absorbed with mouse serum, and with this same immune serum absorbed in addition with a preparation of rat γ globulin purified by passage on a DEAE-cellulose column (Sober and Peterson, 1958). These pictures reveal that the sera contain four to six specific rat antigens in variable quantity. One of these constituents is undoubtedly the γ globulin because a long line

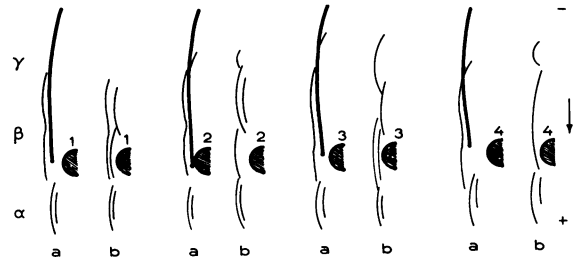

FIG. 2. Immunoelectrophoretic analysis of sera of four mouse/rat chimaeras bled 42 days after irradiation:
(*a*) Analysis with rabbit anti-rat serum absorbed with normal mouse serum;
(*b*) Analysis with rabbit anti-rat serum absorbed with normal mouse serum and with rat γ globulin.

(which characterizes the γ globulins) disappears when one uses an immune serum absorbed with γ globulin. At present, we cannot completely exclude the possibility of erythrocyte proteins having been revealed in some analyses (Popp and Smith, 1959). However, only a small proportion of the experimental sera showed an appreciable degree of haemolysis. Care was taken, moreover, to use only clear non-haemolysed sera for immunizing the rabbits. Rose, Peetom, Ruddy, Micheli and Grabar (1960) have shown that, at least in man, erythrocyte constituents and serum constituents do not share common antigens. Thus, even if small amounts of erythrocyte proteins were present in

some of the serum samples, it is very unlikely that any would have been precipitated by antiserum in the immunoelectrophoretic analyses. Certainly they could not have given rise to a long and prominent precipitation line such as characterizes the γ globulins. The other constituents can be classified only on the basis of their mobility, because we have

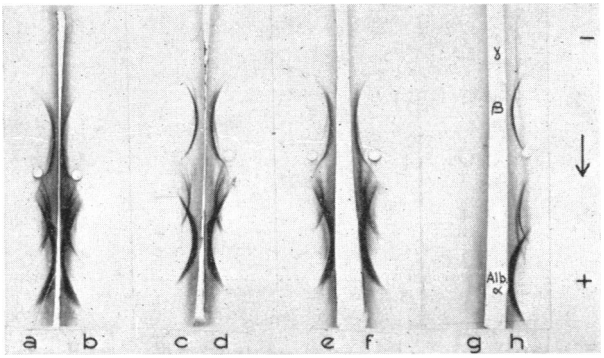

FIG. 3. Immunoelectrophoretic analysis of sera using rabbit anti-mouse serum absorbed with normal rat serum:
(*a*)–(*f*) Sera of mouse/rat chimaeras bled 27 days after irradiation;
(*g*) Normal rat serum;
(*h*) Normal mouse serum.

not tried to define their nature precisely. Two constituents have the mobility of α_2 globulins, one or two of β_1 globulins and at least one of β_2 globulin. Sometimes the arcs of precipitation are not very pronounced and sometimes they are elongated or confluent and, for that reason, more difficult to classify.

As we do not yet possess a sufficiently accurate knowledge of the constituents of rat serum, we prefer for the moment to limit ourselves to considering the γ globulins.

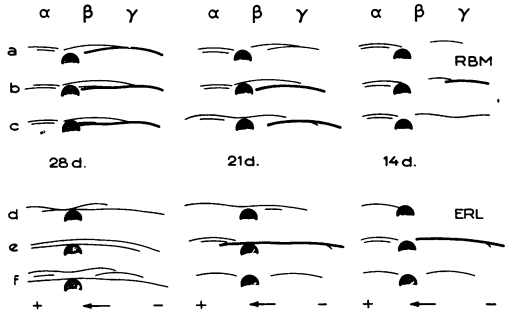

FIG. 4. Immunoelectrophoretic analysis of chimaera sera, taken at weekly intervals after irradiation, using rabbit anti-rat serum absorbed with normal mouse serum:
(*a*)–(*c*) Sera of mouse/rat chimaeras formed with adult rat bone marrow;
(*d*)–(*f*) Sera of mouse/rat chimaeras formed with embryo rat liver.

Fig. 3 shows that established chimaeras contain no γ globulins of mouse specificity or only such quantities as are below the threshold of sensitivity of our method. Anti-mouse immune serum absorbed with normal rat serum gives a line of precipitation characteristic of γ globulin with normal mouse serum, but gives no line of precipitation with normal rat serum (Fig. 3); this shows that the absorbtion has been complete. The absorbed anti-mouse serum gives a large number of precipitation lines with the sera of chimaeras, but

the γ globulin line is never present; tests of six chimaera sera are shown in Fig. 3. These results show that most of the constituents of chimaera sera remain of mouse specificity, but that mouse γ globulin is absent; this confirms our previous observations (Grabar *et al.*, 1957).

Fig. 4 shows that the appearance of constituents of rat specificity in the chimaeras is progressive. This is particularly clear for the γ globulins of which the precipitation line is very feeble, or even absent, 14 days after the injection of marrow, stronger after 21 days and very prominent after 28 days.

In the non-irradiated controls, no rat proteins were detected at any stage.

EXPERIMENT A2

A short-term experiment was undertaken to trace rat proteins in the serum of irradiated and non-irradiated mice injected with large doses of rat bone-marrow cells.

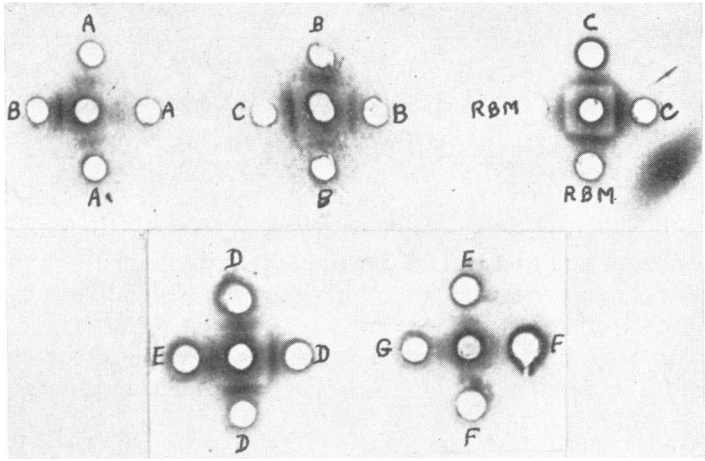

FIG. 5. Identification of rat protein in sera of mice injected with rat cells, using double diffusion in agar. Middle wells contain rabbit anti-rat serum absorbed with normal mouse serum.

Peripheral wells:

A. Sera of mice irradiated and injected i.p. with 10^8 unwashed rat bone-marrow cells. Bled 30 minutes after injection;
B. Sera of mice irradiated and injected i.p. with 10^8 unwashed rat bone-marrow cells. Bled 60 minutes after injection;
C. Sera of mice irradiated and injected i.p. with 10^8 unwashed rat bone-marrow cells. Bled 120 minutes after injection;
D. Sera of non-irradiated mice injected i.v. with 10^7 unwashed rat bone-marrow cells. Bled 60 minutes after injection;
E. Sera of mice irradiated and injected i.v. with 10^7 unwashed rat bone-marrow cells. Bled 24 hours after injection;
F. Sera of mice irradiated and injected i.v. with 10^7 unwashed embryo rat liver cells. Bled 24 hours after injection;
G. Serum of non-irradiated mouse injected i.v. with 10^7 unwashed rat bone-marrow cells. Bled 24 hours after injection.

R.B.M. = suspension of unwashed rat bone-marrow cells in Tyrode's solution.

Two groups of CBA♂ mice, each consisting of four lethally irradiated and four non-irradiated animals, were used. The mice of one group received 10^8 normal male rat bone-marrow cells intraperitoneally and of the other group 1.3×10^7 cells intravenously. Individuals of the former group were bled ½, 1 and 1½ hours after injection, and of the latter group ¼, ½ and 1 hour after injection.

This experiment was done to determine the speed of appearance and disappearance of constituents of rat specificity in the sera of mice receiving rat bone marrow. The sera were examined by Ouchterlony's method of double diffusion in agar. Fig. 5 shows some of the results obtained. At least one constituent of rat specificity can be observed. This constituent appears more rapidly in the sera of mice injected by the intravenous route than in the sera of mice injected intraperitoneally. Analyses made of sera taken 24 hours after injection show only traces of this constituent (Fig. 5).

We were able to demonstrate, using a double-diffusion test and rat serum albumin as reference, by immunoelectrophoretic analysis, and by the technique of Ossermann (1960) using a supplementary trough, that the rat constituent is in fact serum albumin. Moreover when, in a supplementary experiment, we injected mice with a suspension of bone-marrow cells previously washed in Tyrode's solution, there was no longer any evidence of rat constituents in their serum. It may be concluded that the rat constituent or constituents in the serum of mice during the first hours after injection result from contamination of the bone-marrow suspension with rat serum.

As a final confirmation that the rat proteins which we found in the serum of these chimaeras did not result from prolonged persistence of constituents of the injected marrow, we used immunoelectrophoresis to examine extracts of the bone marrow. For this purpose, suspensions of bone-marrow cells were washed and then disintegrated by ultra-sound or by freezing and thawing. The extracts obtained were analysed with anti-rat serum. The results showed that to obtain visible arcs of precipitation it was necessary to use a quantity of extract equivalent to the entire amount of cells used to inject one mouse. In our experiments the constituents of the injected bone marrow were diluted in the whole mouse and consequently could not have been distinguished in the small amounts of these animals' sera which were analysed.

EXPERIMENT A3

Thirty male CBA mice were used. Twenty were irradiated with 1000 rads and ten of these received no further treatment. The other ten irradiated mice and the ten non-irradiated mice received equal amounts (1.6×10^7 cells/mouse) of rat bone-marrow cell suspension which had been subjected to 10,000 rads.

The sera of these animals were taken at various times after injection — 1 to 35 days in the non-irradiated group and 1 to 7 days in the irradiated groups (these animals being all dead 10 days after treatment). Analysis of these sera never showed the presence of any rat constituent.

EXPERIMENT A4

This experiment was designed to show the correlation between the presence of rat γ globulin and the presence of dividing rat myeloid and lymphoid cells in chimaeras. Twenty-one chimaeras of various ages were killed. Their sera, obtained at the time of killing, were studied by immunoelectrophoresis and the proportion of rat dividing cells in bone marrow and spleen was determined.

The results are presented in Table 1. Dividing rat cells and rat γ globulins were only found regularly after doses of 950 rads or above. With one exception (in an animal killed only 16 days after irradiation) rat cells were always associated with the presence of rat γ globulins and *vice versa*.

EXPERIMENT B

In this experiment we used, in parallel with injections of bone marrow, injections of rat foetal liver cells. Twenty CBA♂ mice were irradiated with 1000 rads. Ten of these were injected intravenously with 2×10^7 rat bone-marrow cells each, the other ten each receiving 2×10^7 embryo rat liver cells. A like quantity of rat bone-marrow cells was also injected into ten non-irradiated mice.

The sera of these animals were taken 1, 3, 7, 14, 21, and 28 days after treatment and submitted to immunoelectrophoretic analysis. Only after the 14th day could any constituents of rat specificity be seen in the sera. Fig. 4 is a schematic comparison of the

TABLE 1

CORRELATION BETWEEN PRESENCE OF DIVIDING RAT CELLS IN THE BONE MARROW AND SPLEEN AND PRESENCE OF RAT γ GLOBULIN IN THE SERUM OF MOUSE/RAT CHIMAERAS

Mouse	*Radiation dose*	*Bone-marrow donor*	*Time of when killed (days after irradiation)*	*Dividing cells*		*Rat γ globulin*
				Rat	*Mouse*	
A	850 rads	Rat	128	0	500	−
B	,,	,,	132	0	279	−
C	,,	,,	233	0	63	−
D	,,	,,	369	354	11	+
E	900 rads	,,	237	0	127	−
F	950 rads	,,	49	61	172	+
G	,,	,,	49	50	4	+
H	,,	,,	49	157	148	+
I	,,	,,	56	5	119	+
J	,,	,,	64	183	144	+
K	,,	,,	64	321	1	+
L	,,	,,	64	348	0	+
M	,,	,,	68	219	1	+
N	,,	,,	68	259	4	+
O	,,	,,	69	242	4	+
P	,,	,,	69	67	0	+
Q	,,	,,	531	95	0	+
R	,,	,,	531	94	12	+
S	1100 rads	,,	63	105	1	+
T	950 rads	Mouse/rat chimaera	24	0	14	−
U	,,	,, ,,	16	84	0	−
V	None	—	—	—	—	−

results obtained. It seems that the injections of foetal liver cells gives almost, but not precisely, the same result as the injection of adult rat marrow. However, more experiments would be necessary to obtain precise information.

EXPERIMENT C

Considering the possibility of discerning extra constituents of rat specificity, we performed a number of experiments on mouse/rat chimaeras grafted with pieces of rat skin, thymus or spleen. In most cases grafts of skin persisted until the animal was killed, but those of spleen and thymus did not. These grafts were made at various times after irradiation (Tables 2 and 3).

All the animals so treated and several controls were killed after various intervals, and their sera submitted to immunoelectrophoretic analysis (Figs. 6 and 7).

TABLE 2

DETAILS OF MOUSE/RAT CHIMAERAS GRAFTED WITH RAT SKIN. THE IMMUNOELECTROPHORETIC PATTERNS OF THESE ANIMALS' SERA ARE SHOWN IN FIG. 6. ALL MICE RECEIVED 1000 RADS EXCEPT 2–3, TO WHICH THE DOSE WAS 950 RADS

Mouse No.	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*
Interval between irradiation and skin grafting (days)	97	97	14	14	29	29	29	29	29	29	27	27	27	27	27	27	27	27	27
Survival of skin graft (days)	>434	170–220	>29	>29	>14	>14	7	>18	>18	>18	>20	>20	>20	>20	~16	~20	>21	>21	~20
Interval between skin grafting and killing (days)	434	434	29	29	14	14	14	18	18	18	20	20	20	20	21	21	21	21	21
Condition of mouse when killed	Wasted	Fair	Poor	Poor	Fair	Fair	Poor	Fair	Fair	Fair	Good	Fair	Good	Fair	Fair	Fair	Fair	Fair	Fair

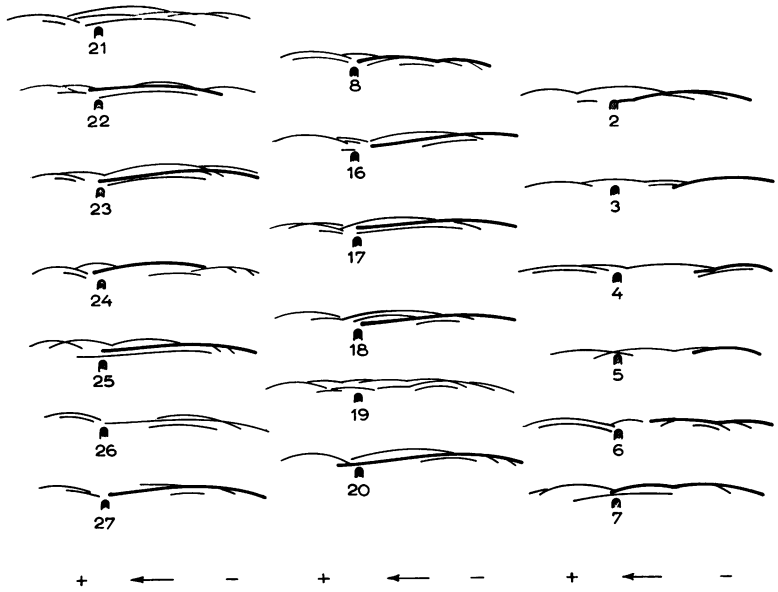

FIG. 6. Immunoelectrophoretic analysis of sera of mouse/rat chimaeras grafted with rat skin, using rabbit anti-rat serum absorbed with normal mouse serum.
For details of chimaeras and grafts, see Table 2.

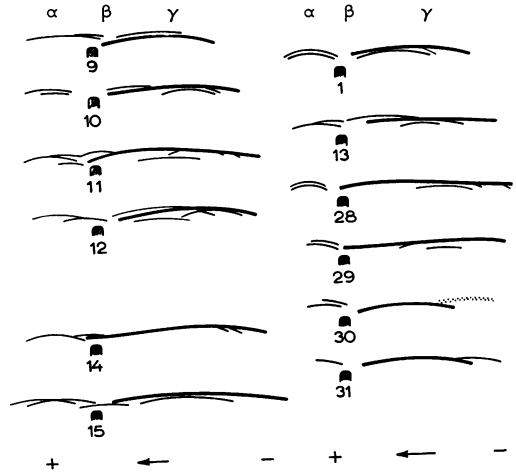

FIG. 7. Immunoelectrophoretic analysis of sera of mouse/rat chimaeras, using rabbit anti-rat serum absorbed with normal mouse serum.
Mice Nos. 9–12 grafted with rat thymus; 14–15 grafted with rat spleen; remainder not grafted. For further details see Table 3.
The dotted lines (Mouse No. 30) represent very feeble arcs of precipitation.

Generally speaking, the number of rat constituents in chimaeras grafted with rat skin was greater than in those that received bone marrow only. The animals which were killed 3 weeks or less after grafting (the majority) tended to have a greater number of distinct rat proteins than those killed after longer intervals (Nos. 2 to 5; Table 2 and Fig. 6); up to ten proteins were discernible. The sera of mice Nos. 2 to 5 approximated more closely to those of non-grafted controls (Table 3 and Fig. 7); mouse No. 3 had shed its graft more than 200 days before it was killed, but Nos. 2, 4 and 5 had intact and hairy grafts. The number of animals grafted with rat thymus or spleen was too small to permit any firm interpretation, but mice Nos. 11 and 12 (Table 3 and Fig. 7) at least seemed to possess some extra rat components.

TABLE 3

DETAILS OF MOUSE/RAT CHIMAERAS WHICH RECEIVED SUBCUTANEOUS GRAFTS OF THYMUS OR INTRAPERITONEAL GRAFTS OF SPLEEN, AND OF NON-GRAFTED CONTROLS. THE IMMUNOELECTROPHORETIC PATTERNS OF THESE ANIMALS' SERA ARE SHOWN IN FIG. 7. ALL MICE RECEIVED 1000 RADS EXCEPT NO. 1, TO WHICH THE DOSE WAS 950 RADS

Mouse no.	*1*	*13*	*28*	*29*	*30*	*31*	*9*	*10*	*11*	*12*	*14*	*15*
Interval between irradiation and grafting (days)							21	23	23	23	34	34
Tissue grafted	None	None	None	None	None	None	Thymus	Thymus	Thymus	Thymus	Spleen	Spleen
Visible persistence of graft to day of killing							−	−	−	−	−	+
Interval between grafting and killing (days)							33	33	33	33	13	13
Interval between irradiation and killing (days)	46	47	48	48	48	48	54	56	56	56	47	47
Condition of mouse when killed	Fair	Poor	Good	Good	Fair	Poor	Poor	Fair	Fair	Good	Poor	Poor

It may be significant that the only two mice in the skin-grafted series whose condition when they were killed was classed as good both showed only faint γ-globulin lines. Possibly the lymphoid tissue of these animals had reverted partly to mouse type. (No investigation of mouse proteins was made in this series.) The presence of a macroscopically intact rat skin graft does not argue strongly against this, since the immunological reactivity of the recovering mouse tissue might well be insufficient to effect rapid breakdown of rat skin, or might indeed be immunologically tolerant of rat antigens. As has recently been shown (Micklem, 1962), rat skin grafts may be retained for up to 2 months by certain mouse/mouse chimaeras.

DISCUSSION

1. The results of our experiments confirm and amplify the conclusion of Grabar *et al.* (1957) that in the chimaeras several serum proteins possess rat specificity. Among these proteins are found γ globulins which can be identified by specific absorption.

Our experiments show that the presence of these proteins is not due simply to the persistence of constituents of the injected marrow. The rat proteins detected in the mouse serum during the first hours after the injection of bone marrow are mainly serum albumin; they are due to the presence of serum in the injected cell suspension. These proteins disappear rapidly from the blood of the mice. On the other hand, the proteins of rat specificity which are detected later in the chimaeras appear only slowly. Their presence, and particularly the presence of γ globulins, can be detected about 2 weeks after the injection of marrow; they increase progressively in quantity and may persist for more than 500 days after the injection of marrow, provided that the rat graft persists. They must be synthesized by the cells derived from the rat bone marrow which have repopulated the reticuloendothelial system of the mice.

Gengozian (1959) criticized our previous results and those of Weyzen and Vos (1957), on the grounds that the use of rabbit or chicken antisera involved a risk of cross-reactions between mouse and rat proteins. But for this, it would hardly seem necessary to emphasize that in all our experiments the rabbit anti-mouse and anti-rat sera were fully absorbed with rat and mouse sera respectively. Improperly absorbed sera would of course be useless.

In his experiments, Gengozian used mouse anti-rat immune sera which made absorption unnecessary. But it is known that sera of this kind are usually weaker in antibodies than sera of immunized rabbits. The fact that Gengozian was not able to detect rat proteins in the serum of chimaeras could thus be due simply to the absence in his immune sera of antibodies against the rat proteins present in the serum of chimaeras. Gengozian does not say that his immune sera actually contained antibodies against rat γ globulin. Our experiments show beyond doubt that the sera of our chimaeras contain γ globulins of rat specificity.

Gengozian's observation that, as a consequence of irradiating his chimaeras a second time, rat proteins appeared in their serum, indicates that rat cells persisted in his animals. As we have shown, the quantity of rat constituents injected initially would be insufficient to be detected in the serum by immunochemical methods because of their dilution in the whole animal. The destruction of rat cells in these chimaeras by the second X-irradiation might liberate some globulins, for which corresponding antibodies existed in Gengozian's immune serum, in quantities sufficient to be detectable.

Although in various experiments (Ford, Ilbery and Loutit, 1957), considerable replacement of the rat graft by mouse cells sometimes occurred (cf. Popp and Smith, 1959), here even long-term survivors had a predominance of rat cells in the bone marrow and spleen, and rat γ globulin in the serum.

2. There was no evidence that γ globulins of mouse specificity were being synthesized in our chimaeras although it may be that they were present in amounts below the sensitivity of our method. The simplest explanation would be that the mouse cells capable of synthesizing γ globulins were totally destroyed by the irradiation. However, certain observations (Ford, Ilbery and Loutit, 1957) have been interpreted as showing that, even after lethal irradiation, regeneration of the lymphoid tissues of the irradiated animal may take place at some later stage. If this is true, the absence of mouse γ globulins in the serum of these chimaeras could be due to some other cause. It might be imagined that the γ globulins synthesized by the regenerating tissue would be entirely anti-rat antibody and would thus be immediately absorbed by the antigens of rat cells still present. However, all that we know of the relation between the formation of γ globulins and of antibodies seems to show that even in an animal during immunization, when there is abundant

formation of antibodies, a considerable quantity of γ globulins which do not possess the properties of these antibodies is also formed. In that case, any γ globulins of mouse specificity formed in the chimaeras would include a proportion which was not absorbed by rat antigens and which should, therefore, have been detected in our experiments.

We do not think that we have the right to conclude that in our mouse/rat chimaeras there are no mouse cells capable of synthesizing antibodies. Although our experiments show that the γ globulins of the chimaera sera are of rat specificity and that they include no detectable amount of mouse γ globulin, this does not exclude the possibility that some antibodies against rat could have been formed (cf. Gengozian, Carter and Peterson, 1961). Exact knowledge of the nature of various antibodies formed by mice is not yet available, but we know that in man antibodies are found not only among the 'family of γ globulins' (Grabar, 1954), but also in the β_2-macro-globulins and probably in the β_2A globulins (Grabar and Burtin, 1960). In mice, relatively large numbers of antigenically different constituents are found in the mobility zones of β and γ globulins (Grabar and Courcon, 1958). In our immunoelectrophoretic analysis of chimaera sera, reacting with anti-mouse sera, we do not observe constituents of slow mobility, that is to say in the zone of γ globulins (or γ_2 globulins of some authors). We do, however, find some mouse constituents in the zone of the β globulins, and one in particular of which the electrophoretic position could be likened to the β_2A-globulin position in man (Fig. 3). If the mouse is capable of synthesizing antibodies which are antigenically distinct from γ globulins, it is not impossible that our chimaeras are capable of the formation of such antibodies. This hypothesis would explain some discrepancies between our observations and those of other authors.

The relative importance of cellular and humoral reactions in the immunological rejection of an established bone-marrow graft are not as yet known. The rejection of first-set skin grafts appears to be primarily cellular and analogous to hypersensitivity of the delayed type (Brent, Brown and Medawar, 1959). On the other hand some tumours are highly susceptible to humoral antibody (Gorer, 1958), as are bone-marrow cells injected into passively immunized mice (Loutit and Micklem, 1961). Micklem and Brown (1961) showed that humoral iso-antibody production could be suppressed by radiation at a dose insufficient to affect the rejection of foreign skin homografts. Consequently our observation that the γ globulins, and hence the majority of antibodies of classical type, are synthesized in chimaeras by rat cells cannot completely rule out the possibility of immunological activity by the host against the graft. Nevertheless, we consider that the syndrome of 'secondary disease', from which many of the animals in these experiments suffered, was set in motion in this, as in many other situations, by an immune reaction by the graft against the host.

3. Our experiments also provide some information concerning the origin of serum proteins. On the basis of simple electrophoretic methods, only serum γ globulins have generally been agreed to originate from cells other than those of the liver. Immunoelectrophoretic analysis has enabled us to distinguish several rat proteins in the serum of chimaeras, including animals in which the state of chimaerism had been long established and in which we found all, or the great majority, of dividing myeloid and lymphoid cells to be of rat karyotype. These proteins must be synthesized only by cells derived from the rat bone marrow. Some of our experiments involving the injection of embryonic rat liver cells and the skin-grafting of chimaeras suggest that these grafted cells are capable of synthesizing serum constituents different from those produced by bone-marrow cells and

their derivatives. We detect the proteins with an antiserum against normal rat serum so they must be proteins antigenically similar to proteins normally present in rat serum. It is just possible that the extra rat constituents seen in animals grafted with skin result from the breakdown of cells in the graft due to slow initial healing or to a niggling immune reaction by the host, but the results of Experiment A2 and A3 make such an explanation seem very unlikely. Even if portions of the graft were breaking down, the products of the breakdown would not be sufficient in quantity to produce such numerous and easily distinguished proteins in the serum. The suggestion that some normal serum proteins may be formed in the skin seems not unreasonable, although it is in conflict with the widely accepted, but perhaps over-simple, view of the liver's predominant role in the synthesis of serum proteins. Further experiments are being done to resolve the question.

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