without being forced to rely on a single criterion. Wild-type and non-mutant protein could be compared to see whether all determinants of both parents are possessed by the new protein. Also, were the molecules capable of dissociation into sub-units retaining their antigenicity, specific sub-units might be recognized by their determinant characteristics. The time of appearance, localization and concentration changes of sub-units could then be followed. A description of the course of assembly of native proteins during development might also prove practicable at a finer level than previously attempted. A related area of considerable interest which would probably benefit from detailed analysis of sub-unit reassortment and aggregation is that of the control of the synthesis of isozymes[11].

In conjunction with other methods, it may also be possible for this technique to be useful in resolving certain problems of protein structure not otherwise easily attacked. For example, *Neurospora* adenylsuccinate synthetase mutants located at certain points within a cistron tend to restore enzymatic activity to a degree greater or less than would be expected based on their position within the linkage group[12]. Were a refolding of a polypeptide chain or a juxtaposition of sub-units responsible for a return to a near-native configuration[13], then conceivably the determinant construction of heterokaryons might be more like the wild-type than either parent separately or in combination—that is, a 'new' antigenic specificity might be found. The lack of agreement between the linearity of linkage and complementation maps possibly could be resolved were concomitant changes in determinant composition also known. Since antigenic specificity itself is known to be highly dependent on folding, conclusions about tertiary structure might also be drawn from determinant analysis.

The probability of success in extending this approach to systems other than the *Paramecium* immobilization antigens appears to be high. The *Paramecium* antigens that have been successfully used here are not particularly effective as immunizing agents, rarely eliciting sera with antibody concentrations as high as 200γ per ml. Particularly suitable would be an examination of evolutionary relationships among species and genera through a comparison of individual serum proteins, such as albumins. This, of course, would provide but a single criterion of ancestry and might in itself be misleading. Thus, one determinant difference between two albumins, and two differences between either of these and a third albumin, need not mean that the original albumins are more 'closely' related than is either to the third. The single determinant difference may be a consequence of several amino-acid substitutions, and each of the other determinant differences result from a single amino-acid difference.

The major advantage offered by this immunological approach is that a single parameter is not relied on to compare proteins. For example, a change in net charge that is a reflexion of a single amino-acid substitution may also be mimicked by the sum of several amino-acid changes. However, using the suggested antigenic analysis, the number of parameters is greatly increased as each determinant may be followed separately, and a single net effect is less likely to obscure several independent alterations.

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RETENTION OF IMMUNOLOGICAL INFORMATION

By STAFF OF THE CHESTER BEATTY RESEARCH INSTITUTE, ROYAL CANCER HOSPITAL, LONDON, S.W.3

(I) By Syngeneic Radiation Chimæras

By DR. A. J. S. DAVIES, BARBARA DOE, A. MARJORIE CROSS and E. V. ELLIOTT

IT is recognized that immunological information developed in an adult mouse in response to antigenic challenge may be retained when the animal is irradiated and given syngeneic bone marrow but is not retained to the same extent after irradiation and injection of allogeneic bone marrow[1,2].

A further series of experiments concerned with the immune responses of syngeneic and allogeneic radiation chimæras to sheep red cells is here described. The results are examined in order to determine whether residual immunological activity after irradiation and bone marrow therapy must necessarily be attributed to persisting host cells.

Mice

Male mice of the Chester Beatty *CBA* and *BALB/c* and of the Harwell *CBA/T6T6* strains were used throughout. Host (*CBA*) mice were 10–15 weeks of age at the time of irradiation. Donor mice (*BALB/c* or *CBA/T6T6*) were 8–10 weeks of age when killed. The *CBA/T6T6* mice have marker chromosomes[3].

Immunization

(*a*) *Active*. Mice were immunized by intraperitoneal injection of 0·2 ml. of a 20 per cent suspension of sheep red blood cells in Alsever's solution, 20 and 13 days before irradiation and 60 days after irradiation.

Immune mice were bled from the retro-orbital sinus. Serial doubling dilutions were made of the serum in saline in 'Perspex' hæmagglutination tiles. Aliquots of thrice-washed sheep red cells were added to the tiles, which were kept at 37° C for not less than 2 h. The total titration volume was 0·05 ml. Titres were expressed as logarithms to the base two of the reciprocal of the final dilution showing macroscopically visible agglutination.

(*b*) *Passive*. Eighty inbred male *BALB/c* mice were immunized with sheep red cells on each of three occasions separated by a fortnight. Seven days after the third injection blood was taken from the mice, pooled and after 1 h at 4° C, to facilitate retraction of the clot, the immune serum was taken off.

0·4 ml. of this serum was injected intraperitoneally into each of 48 male *BALB/c* mice. Titrations were carried out on individual sera from all passively immunized mice 36 h, 6·5, 13·5, 20·5 and 27·5 days after their injection with immune serum. In all instances about 0·05 ml. of blood was withdrawn from the retro-orbital sinus. This afforded the minimum amount of serum that could be used in our titration system and represented about one-fortieth of the total blood volume of the mice. In these circumstances as only five bleedings were made on any one mouse it was felt that any effect of bleeding *per se* on serum titre could be ignored. The passively immunized mice were split into three groups of of 16 (four boxes with four mice in each). Two of these groups were subjected to 850-r. X-irradiation three days after passive immunization. One group of irradiated mice was injected intravenously with 10×10^6 cells of syngeneic (*BALB/c* bone marrow, the other with the same number of allogeneic (*CBA*) bone marrow cells, within 4 h of irradiation. The third, unirradiated group of mice was not subjected to further treatment. The irradiated mice would have died had they not been injected with bone marrow.

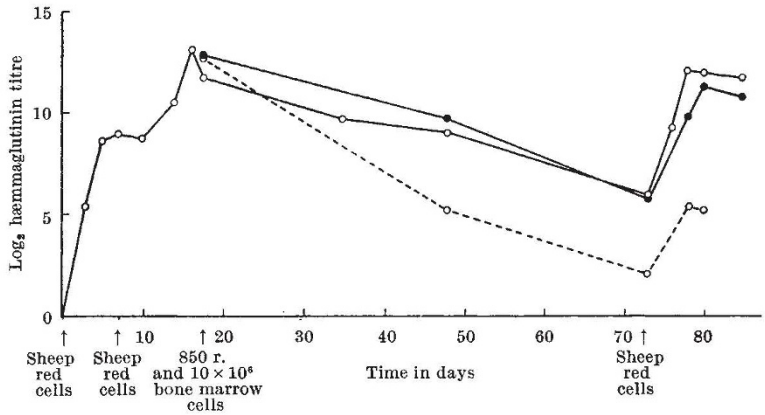

Fig. 1. The hæmagglutinin titres of various kinds of *BALB/c* mice in relation to time. (The normal mice were not irradiated)
—○—, Normal mice; —●—, *CBA/T6T6*→*CBA* syngeneic chimæras;
– – –○– – –, *BALB/c*→*CBA* allogeneic chimæras

Irradiation and Bone Marrow Therapy

All mice received 850-r. total body dose at LD_{99}, 30 days and within 5 h were given, intravenously, 10×10^6 cells of either *BALB/c* (allogeneic) or *CBA/T6T6* (syngeneic) bone marrow in 0·4 ml. medium 199. Mice were maintained for 5 days before irradiation and 15 afterwards on a 20 per cent protein diet containing aureomycin (in the form of 'Aurofac' kindly supplied by the Cyanamid Chemical Corp., Ltd.; ostimated dose 1 mg/mouse/day).

Cytological Check on Chimærism

Three and five days after receiving their third injection of sheep red cells (that is, 63 and 65 days after irradiation and bone marrow injection) groups of chimæras were injected with 'Colcemid' (4 mg/kg body-wt.) $1\frac{1}{2}$ h prior to removal of half the spleens under ether anæsthesia. The piece of spleen removed was divided into two, half being retained for histological examination and half being made into a cell suspension in hypotonic sodium citrate preparatory to cytological examination[4] (syngeneic chimæras only).

Serological Check on Chimærism

Sixty days after irradiation red cells were removed from putative allogeneic chimæras and tested for their identity with appropriate iso-antisera[5].

Tests for Chimærism

(1) *Allogeneic chimæras.* All 20 animals surviving until 60 days after irradiation were found to have exclusively *BALB/c* (donor) red cells.

(2) *Syngeneic chimæras.* 289 cells in nine mice engaged in a response to a third injection of antigen were examined and were found all to be of donor (*CBA/T6T6*) type. This result agrees with the larger series of experiments[6] showing that host cells are rarely found 30, 50 or 100 days after 850-r. irradiated *CBA* mice have been injected with *CBA/T6T6* bone marrow.

Histology

Portions of the spleen removed on the third or fourth day after injection of sheep red cells (63 or 64 days after irradiation) were fixed and prepared for histological analysis. It was not possible to distinguish on the grounds of mitotic frequency between animals undergoing a primary and a secondary response to sheep red cells.

Titrations

(*a*) *Active immunity.* The titration results are given in Table 1 and Fig. 1. It can be seen that the response of normal *CBA* mice to a single injection of sheep red cells is rapid, giving a peak titre within 5–7 days. The primary response of syngeneic chimæras 60 days after irradiation (not shown in Fig. 1) was very similar and the peak titre was not significantly different from that of normal mice. The primary response of the allogeneic chimæras 60 days after irradiation was, however, significantly less than either normal mice or syngeneic chimæras.

The response of normal mice to a second injection of sheep red cells was also found to be rapid, giving a peak titre about 7 days after injection which was about 3–4 logs above the peak following the first injection. The titration values of the mice which were to be irradiated showed that they were behaving as normal before irradiation and could be regarded as satisfactory experimental material. It is noteworthy that titres observed in the syngeneic chimæras 41 and 66 days following the last contact with sheep red cells almost exactly paralloled those found in normal mice over the same period. In contrast the titres in the allogeneic chimæras were significantly lower than those of normal mice and of syngeneic chimæras.

The response of normal mice to a third injection of sheep cells was more rapid than that of the syngeneic

Table 1. Log_2 HÆMAGGLUTININ TITRES (±*S.D.*) OF VARIOUS KINDS OF *CBA* MALE MICE

Response to injection No.	Days after last injection of antigen	Normal mice	*CBA/T6T6* →*CBA* chimæras	*BALB/c* →*CBA* chimæras
1	3	5·43 ± 1·25	—	—
	5	8·75 ± 0·83	9·67 ± 0·92*	—
	7	9·00 ± 1·00	9·33 ± 0·95	5·14 ± 1·63*
	12	—	9·00 ± 1·0	—
2†	3	8·75 ± 0·97	—	—
	5	10·50 ± 1·80	—	—
	7	13·06 ± 2·92	—	—
	12	11·71 ± 0·71	(12·75 ± 0·76)‡	(12·75 ± 0·76)‡
	28	9·73 ± 1·62	—	—
	41	9·00 ± 0·50	9·78 ± 1·44	5·13 ± 1·03
	66	5·87 ± 0·35	5·67 ± 1·28	2·00 ± 0·73
3§	3	9·25 ± 0·66	—	—
	5	12·12 ± 0·65	9·83 ± 1·75	5·36 ± 1·90
	7	12·00 ± 0·50	11·33 ± 3·30	5·23 ± 1·48
	12	11·75 ± 0·66	10·78 ± 1·77	—

* Injection 60 days after irradiation.
† Injection seven days after first injection.
‡ Titres two days before irradiation.
§ Injection 73 days after second injection, 60 days after irradiation in the case of chimæras.

Table 2. DECLINE IN PASSIVE IMMUNITY OF THREE CLASSES OF *BALB/c* MICE EXPRESSED AS MEAN FALLS IN TITRE AND CALCULATED HALF-LIVES BETWEEN THE TIMES INDICATED AFTER PASSIVE IMMUNIZATION

Mice	Mean drop in titre and calculated half-lives during period indicated (see Fig. 2)							
	Period 1		Period 2		Period 3		Period 4	
	Mean drop in titre	Half-life	Mean drop in titre	Half-life	Mean drop in titre	Half-life	Mean drop in titre	Half-life
Normal	2·38 ± 1·45	2·10	1·50 ± 1·06	4·67	1·47 ± 0·89	4·76	1·20 ± 0·72	5·83
Syngeneic chimæras	2·86 ± 0·63	1·75	1·64 ± 0·48	4·27	2·00 ± 0·71	3·50	1·00 ± 0·93	7·00
Allogeneic chimæras	3·25 ± 1·09	1·54	2·88 ± 0·77	2·43	1·63 ± 0·85	4·29	1·50 ± 0·50	4·67

chimæras, but the peak titres were not significantly higher. In both cases the peaks reached about the level attained during a response of normal mice to a second injection of red cells. At twelve days the response of the syngeneic chimæras to a third injection was significantly higher than the response of a similar group of mice to a first injection of red cells.

The response of the allogeneic chimæras to a third injection was indistinguishable from a primary response and was in any case drastically reduced in comparison with either normal mice or syngeneic chimæras.

(*b*) *Passive immunity.* The results of the passive immunity experiment which are summarized in Fig. 2 and Table 2 can be interpreted as follows: the titre of the pooled immune serum was $\log_2$ 13 before its injection into the experimental mice. Within 36 h the serum titres of the injected mice were about $\log_2$ 11. This drop in titre could be attributed largely to dilution, but perhaps also to retention of antibody in extravascular sites and in part to decay or destruction of the antibody molecules. In normal mice the rate of decline of amount of antibody does not vary significantly after 36 h and follows an exponential curve which defines the half-life as about 5 days. Both groups of irradiated mice show a significantly more prolonged period of accelerated decay than normal mice, but stability is achieved in the syngeneic chimæras during period one (top of Fig. 2) and in period two in the allogeneic chimæras.

Statistical analysis showed that there were no significant differences between the decay rates of antibody in period one taken by itself. During period two, however, the two groups of chimæras differed from each other, the allogeneic having a higher decay rate than the syngeneic chimæras. Further, the allogeneic chimæras had a higher decay rate than did the normal mice. Within period three the decay rates were similar for all groups of mice giving an antibody half-life of almost 5 days.

The results of Garver *et al.*[1] and of Hollingsworth[2] taken in conjunction with the present findings demonstrate that active immunity to red cell antigens can persist in heavily irradiated animals that have received syngeneic bone marrow as a therapeutic measure. The additional information provided by the present findings can be assessed after consideration of the results of the subsidiary experiment on passive immunity, which showed that in normal mice and irradiated animals the biological half-life of mouse anti-sheep-red-cell antibody was about 5 days. This value is similar to that of 4·5 days quoted by Humphrey (personal communication), rather higher than that which can be calculated from the results of Mitchison[7] and lower than the 8 days quoted by Dresser[8]. On the basis of this result, Fig. 1 needs the added interpretation that actively immune normal mice and syngeneic chimæras synthesize antibody during the period from day 20 to day 70, as the rate of decline of amount of antibody (apparently half-life 9 days) is too slow to be accounted for simply by passive loss.

The rate of passive decline when allogeneic bone marrow is injected after irradiation indicates an antibody half-life of 2 days for about 8–10 days, after which it approximate to the normal passive decay rate. It is not possible to say whether synthesis of antibody stops or is reduced in the allogeneic chimæras, but it is possible to say that by 20 days after irradiation and probably less it is continuing at approximately the same (declining) rate as was observed in normal mice and syngeneic chimæras, assuming that the antibody present is qualitatively unchanged.

For the present purpose, immunity may be considered under two headings: (*a*) synthesis of antibody; (*b*) the anamnestic response. The synthesizing component is apparently insensitive to radiation and the injection of bone marrow. The anamnestic component is apparently destroyed by irradiation and injection of allogeneic bone marrow but is maintained, albeit slightly reduced, after irradiation and the injection of syngeneic bone marrow.

The most plausible explanation of the results is "that treatment of severe irradiation injury with bone marrow cells does not supplant the humoral antibody production mechanisms of the host with those of the donor"[1]. This may be extended to cover skin homograft immunity on the basis of the results of Cross *et al.*[9]. The question to decide is, first, whether host cell persistence is likely and, secondly, if host cells do persist, what type of cell is required for the maintenance of immunity as considered here.

Early in the history of the investigation of radiation chimæras Ford and his colleagues[10] showed that damaged host cells could be found dividing in the spleen, thymus,

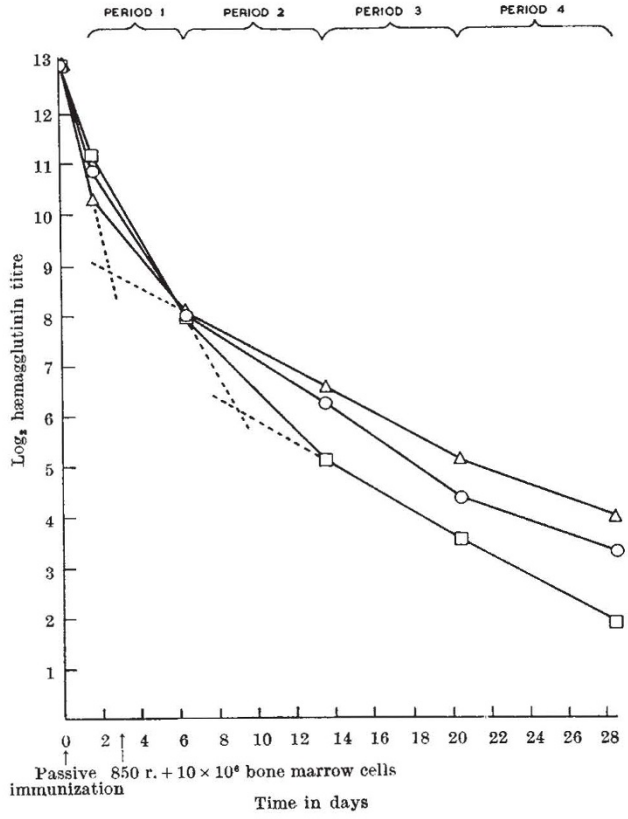

Fig. 2. The hæmagglutinin titres of various kinds of passively immunized *BALB/c* mice in relation to time (normal mice were not irradiated) △, normal mice; ○, *BALB/c*→*BALB/c* syngeneic chimæras; □, *CBA*→*BALB/c* allogeneic chimæras

bone marrow and lymph nodes of irradiated animals injected with bone marrow. These cells were found in diminishing numbers with increasing time after irradiation and could only rarely be found after 30 days. Dividing cells present at later times in all hæmatopoietic and lymphoid organs were almost always donor cells. Since then many methods, other than cytological ones, have shown that chimærism established after radiation is often permanent and complete.

The cytological and serological checks on chimærism used in the present experiments, 60 days after irradiation, indicated that the erythropoietic system of the allogeneic chimæras and the dividing cells in the spleens of the immunologically stimulated syngeneic chimæras were donor type. Thus the chimæras the immune response of which is under consideration were typical, but neither the tests performed on them nor the data about chimæras adduced here are sufficient evidence that no host cells were present. All that can be said is that they were not discovered.

The problem can be approached from another aspect. If the immune response observed is a property of the host, which cells are involved and would they be detectable in tests for chimærism ?

The antibody synthesized by the chimæras and by the normal mice during the decline phase of the immune response is likely to have derived largely if not wholly from plasma cells, as few other cells have been shown regularly to synthesize antibody. It is well known that plasma cells appear after irradiation, though it is not clear whether they simply become obvious because other cells have been destroyed and phagocytozed or whether they are produced in response to an immunological stimulus[11]. Nossal[12] maintains that at least some plasma cells have a relatively short functional life and cell division is required to ensure their replacement. If such divisions of host cells occur in order to provide antibody-producing cells, why have they not been seen ? Alternatively, the plasma cells under consideration are long-lived and radiation-resistant. No conclusion can at present be arrived at except to say that the mechanism for the continuation of antibody synthesis may in part survive irradiation, but even if the host cells observable early after irradiation are part of the mechanism, its cellular components cannot be found to divide in appreciable numbers subsequent to 30 days after irradiation. It is tempting to wonder whether the continuation of antibody synthesis becomes a property of the donor cells, which are present in abundance in radiation chimæras, rather than remaining a property of host cells the very existence of which may be a chimæra.

Nossal[12] concludes from an extensive and elegant series of experiments that "the cell type (in resting primarily immunized nodes) capable of responding instantly to antigenic stimulation, and thus presumably carrying specific immunological memory, is a rapidly proliferating primitive lymphocyte". The experiments of Makinodan *et al.*[13] indicate that the D_{37} of the appropriate cells, primitive lymphocytes or not, is 70 r. The dose of radiation used in the present experiments was 850 r. and it must be concluded that very few of the relevant cells could have survived such irradiation. If, however, sufficient cells did survive and, by their active proliferation on antigenic stimulation, 60 days after irradiation, initiated the response to a third injection of sheep cells observed in the syngeneic chimæras, why were not their divisions observed ?

The allogeneic chimæra proved to be incapable of effecting a recognizable anamnestic response which could either mean that the relevant host cells had been destroyed by a graft-versus-host reaction or that part of the immunological system necessary for the receipt and processing of antigenic material fails to develop. The authors incline to this latter view and are attempting to disprove it.

Over all, the evidence for the persistence of sufficient host cells to maintain immunity in chimæras as observed is not good. The alternative hypothesis, that it is the donor cells which do the work, requires that the capacity to do so is in some way transferred from the irradiated host cells to the incoming donor tissues. This possibility will be considered more extensively elsewhere. It must suffice to say here that we can find no evidence, after irradiation of immune mice, for massive release of antigen, complete in that it simulates injection of sheep red cells (unpublished observations). The finding discussed by Campbell[14], that immunity to protein antigens may be maintained by the persistence of haptenic polypeptides strongly bound to low molecular weight RNA, which, it is not inconceivable, could be liberated on irradiation, is perhaps relevant. Further, there is an accumulation of largely circumstantial evidence that transfer of immunological information between cells is a very real possibility[15–22].

The problem raised here from the very artificial situation existing in radiation chimæras relates to the general problem of the persistence of immunological information in immunized mice. However, until a clear description of the cells involved in an immune response can be made any approach is bound to have a large speculative component. This matter has been thoroughly discussed by Humphrey[23] and it should be noted that he says "we cannot rule out the possibility that the precursors of antibody-producing cells never actually ingest antigen directly, but are stimulated by nucleoproteins, derived from phagocytic cells".

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(II) By Thymectomized Syngeneic Radiation Chimæras

By A. MARJORIE CROSS, ELIZABETH LEUCHARS and Dr. A. J. S. DAVIES

It has been shown[1] that mice which were immunized to sheep erythrocytes, before exposure to a potentially lethal dose of irradiation followed by syngeneic bone marrow therapy, continued to synthesize hæmagglutinins after irradiation. When a further injection of sheep erythrocytes