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# CONGENIC MOUSE STRAINS WITH DIFFERENT IMMUNOGLOBULIN ALLOTYPES

## I. BREEDING SCHEME, HISTOCOMPATIBILITY TESTS, AND KINETICS OF $\gamma G_{2a}$-GLOBULIN PRODUCTION BY TRANSFERRED CELLS FOR C3H.SW AND ITS CONGENIC PARTNER CWB/5[1]

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SUMMARY

The production of congenic lines of mice differing at immunoglobulin loci is described. After five backcross generations a pair of lines was developed which, although skin-compatible, still have barriers for transferred spleen cells, as followed by quantitating donor type γ-globulins in the recipients' sera. The barriers are radiation-sensitive and can be overcome by large numbers of transferred cells. One barrier, which can be largely overcome by 300R to the recipient is described as lack of "biological space." The second barrier may be residual histoincompatibility.

Spleen, bone marrow, and fetal liver but not thymus cells produce γ-globulins for many weeks after injection into 600R-irradiated congenic recipients. As donor type levels increased, host type decreased in some animals to very low levels.

The usefulness of the congenic strains being produced for studies of cell immunological potential, regulation, and function is discussed.

## INTRODUCTION

Congenic lines are defined as strains bred to approach genetic identity except for different alleles at one locus. Snell pioneered the use of such strains in mice for studying the genetics of histocompatibility, and through his efforts we now have available a large number of strains which differ from one another only by a single histocompatibility gene. By thus restricting the genetic differences between individuals to a single locus, its effects can be directly studied independently of other differential genetic influences (*14–16*).

In the belief that congenic lines for immunoglobulins would be useful for all types of cell and organ transfer studies minimizing problems of histoincompatibility, we set out to develop such lines. A series of linked genes for the

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H-polypeptide chain of immunoglobulins (γ-globulins) has been identified in mice by allotypic (isoantigenic or alloantigenic) differences. Our plan was to mate mice of inbred strains with different alleles at the first discovered locus (Ig-1) and repeatedly backcross heterozygotes to one or the other parental strain. In order to speed approach to coisogenicity (the ideal of genetic identity except for the selected allele) mice selected for maximal skin histocompatibility were used for further backcrossing at two stages in the breeding program.

This paper is a progress report on studies with one congenic line. This line, CWB/5, is completely skin compatible with its inbred partner C3H.SW (= CSW).

Cell transfer studies with suspensions from various lymphoid or hematopoietic organs are reported. There are two remaining barriers to permanent acceptance of such transferred cells. However, these barriers are overcome by sublethal irradiation and/or large cell inocula.

## MATERIALS AND METHODS

*Congenic lines.* The congenic lines used in the present experiments were derived from two inbred strains: C3H.SW/SnHz and C57BL/10SnHz, received in 1960 from Dr. G. D. Snell of the Jackson Laboratory, Bar Harbor, Maine. Both these strains have the same H-2 allele ($H\text{-}2^b$) but they differ in non-H-2 histocompatibility loci. They differ also at four immunoglobulin H-chain loci: *Ig-1,2,3,4* (*9, 10, 12*). CSW is $Ig\text{-}1^a/Ig\text{-}1^a$ and B10 is $Ig\text{-}1^b/Ig\text{-}1^b$.

The congenic lines were produced as follows: Strains CSW and B10 were crossed and their $F_1$ progeny backcrossed to CSW. The backcross progeny were typed for Ig-1 phenotype and the $Ig\text{-}1^a/Ig\text{-}1^b$ individuals backcrossed again to CSW. Five successive backcrosses in all were made to the CSW strain. At backcross generations 3 and 5, skin grafts from prospective breeders for the next generation were transplanted to CSW mice of the same sex presensitized with spleen cells and skin grafts from B10 mice. The one individual male of the third backcross generation whose graft was not rejected during a 3-month period was used for further matings. The grafts from the fifth backcross were not rejected during the lives of the recipients. After the fifth backcross the $Ig\text{-}1^a/Ig\text{-}1^b$ mice were intercrossed, the $Ig\text{-}1^b/Ig\text{-}1^b$ progeny selected and set up for continued brother × sister matings.

The strain derived in this fashion, named CWB/5, is congenic with CSW, differing at the *Ig-1* locus. The CWB animals used in the present experiments underwent at least six brother × sister matings in addition to the five backcross matings. These animals should have on the average $1/2^6$ or about 2% of unlinked CSW alleles and a considerable portion of the CSW chromosome region surrounding the *Ig-1* locus. The experimental animals were 2 to 3 months old and were of both sexes.

*Skin grafting.* A slightly modified method of Billingham and Medawar (*3*) was used.

*Irradiation conditions.* A Siemens X-ray machine was the irradiation source. The conditions were: 250 kv., 15 ma, 0.25 mm Cu and 1.0 mm Al, HUL

1.10 mm Cu. The mice were placed into circular perforated plastic boxes at 50 cm distance from the source. The dose rate at the target was 83.5R/min.

*Cell transfer.* Spleen or thymus cell suspensions were obtained by cutting the organs into small pieces with scissors and gently pressing the tissue through a stainless steel screen into Hank's balanced salt solution. The suspensions obtained were aspirated several times through a 25-gauge needle and then counted in a hemocytometer. The viability of the cells was judged by a dye exclusion test (trypan blue).

*Serum collection.* Recipients were bled every 7 or 10 days by cutting the tip of the tail and collecting the blood into capillary tubes. The sera were separated by centrifugation and kept frozen at −20°C until the date of use.

*Inhibition assay.* The γ-globulin level of recipient mice was measured by the inhibition of precipitation of $^{125}$I-labeled γ-globulin. Details of this procedure are described elsewhere (*10, 17*).

*Determination of donor or host type γ-globulin levels.* The inhibition of precipitation of $^{125}$I-labeled antigen was used as previously described (*10, 17*). The values reported are as percentages of a standard serum pool of normal young adult animals of C57BL/6 for Ig-1b and BALB/c for Ig-1a. These have proteins ($\gamma G_{2a}$-globulins) carrying these allotypic antigens at a concentration in the order of 10 mg/ml of serum. As used this method detects 0.05% of the standard of one type in the presence of normal levels of the other allotype.

*$^{125}$I-labeled antigens.* For Ig-1b a fraction of C57BL/6 γ-globulin eluted from a diethylaminoethyl cellulose column between approximately 0.01 M NaCl and 0.03 M NaCl in 0.01 M Tris-HCl buffer at pH 8.1 was used. For Ig-1a a purified myeloma protein produced by the BALB/c plasma cell tumor RPC-5 was used. This tumor was kindly provided by Dr. John Fahey and originally induced by Potter (*13*). $^{125}$I-coupling was as previously described (*10, 17*).

*Isoantisera.* Two isoantisera against γ-globulins were used in the inhibition assay: BALB/c anti-B10 and B10 anti-A/J. The sera were prepared as follows: In the first case B10 mice were immunized against DBA/2 spleen cells and the anti-tissue serum was then injected into BALB/c mice (a primary subcutaneous injection of 10 μl of whole serum per mouse in complete Freund's adjuvant, followed 3 weeks later by two weekly i.p. injections, each of 10 μl). In the second case, A/J mice were injected with B10 spleen cells and the anti-tissue antiserum injected into B10 mice (the same protocol as in the first case).

## RESULTS

*Skin graft test for histocompatibility.* Seven CWB females (3 months old) were chosen as recipients for skin grafts (skin from the back or belly) from CSW female donors. The grafts were observed starting 14 days after transplantation twice a week in the 1st month and once a week in the rest of the period. No sign of rejection was found during the observation period (more than 250 days).

Since it is known that weak histocompatibility barriers are often transgressed by allogeneic tissue, unless the recipient is preimmunized against the

donor (6), we decided to test the histocompatibility of skin grafts in preimmunized recipients. We used the immunization schedule of Graff et al. (6): five CWB females and five CWB males were injected with thymocytes from 1-month-old CSW donors of the same sex (1–3 × $10^6$ thymocytes per mouse, three times at weekly intervals, intraperitoneally) and 7 days after the last injection the skin grafts were applied. The grafts were inspected for more than 150 days once a week, and no sign of their rejection was observed.

The two congenic lines are therefore histocompatible as far as the skin grafts are concerned.

*Cell transfer into nonirradiated recipients.* Thirteen CWB females were injected intraperitoneally with CSW spleen cells (1 × $10^6$ cells per mouse). The sera from the recipients were collected and tested for the presence of donor-type γ-globulin by the inhibition assay. The results are represented by the first curve in Figure 1. The presence of donor type γ-globulin was detected only once during the whole observation period (24 days after injection) and its level was very low (less than 1% of the level in standard serum). Later the donor-type γ-globulin disappeared completely from the circulation of the host animals. These results suggest that the transferred spleen cells are either destroyed in the host or somehow prevented from producing γ-globulin. To test these two possibilities, further experiments were carried out.

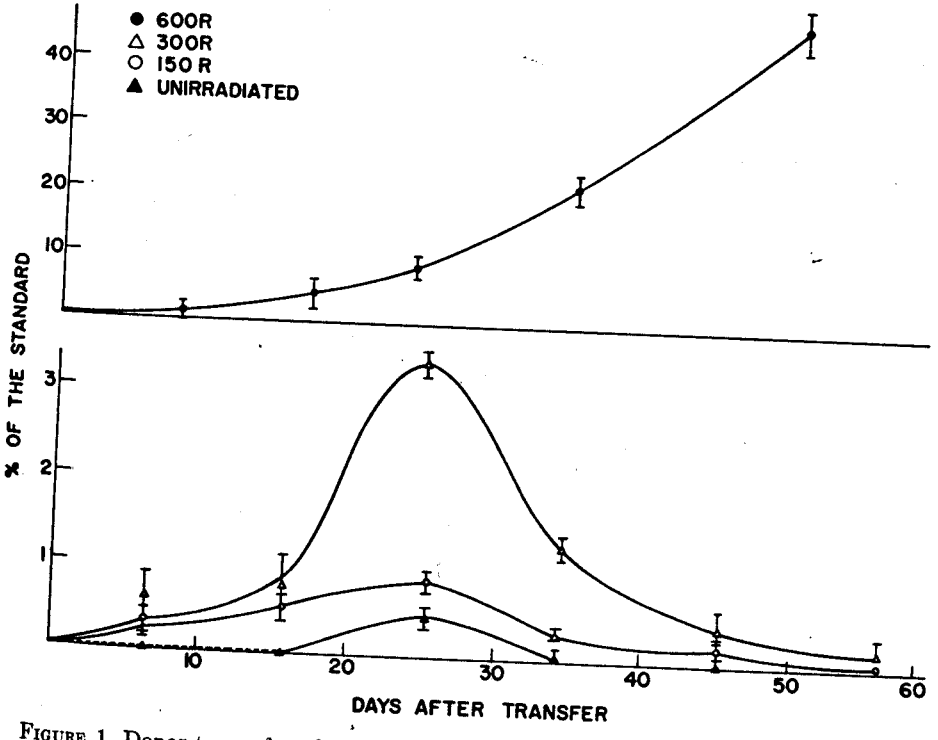


FIGURE 1. Donor-type of γ-globulin level in CWB recipients injected intraperitoneally with CSW spleen cells (1 × $10^6$ cells per mouse). The effect of different doses of irradiation. The vertical bars indicate ± standard deviation in this and subsequent figures.

*Cell transfer into irradiated recipients.* The effect of different doses of X-irradiation was explored first. Twenty-one CWB males were divided into three groups, seven animals in each. The groups were irradiated with 150R, 300R and 600R respectively. The following day all irradiated animals were injected intraperitoneally with spleen cells from CSW male donors ($1 \times 10^6$ spleen cells per mouse). The sera from the recipients were collected and tested for the presence of donor type γ-globulin by the inhibition assay. The results represented by the three curves in Figure 1 show a marked effect of the dose of irradiation of the recipient on the level of γ-globulin produced by transferred cells. (Each point of the curves represents the average from seven individual mice, except the curve for 600R where, because some animals died during the experiment, the experiment was finished with four instead of seven animals). In the animals irradiated by 150R and 300R the peak is reached at about 25 days after cell transfer (less than 1%, and about 3% of the level of standard serum respectively). Later the level in both cases decreased slowly to zero. In animals irradiated with 600R the donor-type γ-globulin level is much higher and increases toward the end of the experiment (almost 50% of the level in standard serum about 55 days after cell transfer).

The next experiments were designed to determine whether there was a more rapid elimination of donor γ-globulin.

*Elimination of labeled antigen.* The CWB recipients were divided into two groups: experimental and control. In the experimental group, animals from the previous two experiments were used—2 unirradiated females, 4 males irradiated with 150R, and 2 males irradiated with 300R all after disappearance of the donor-type γ-globulin in their circulation. Since no significant difference between these three subgroups was found, pooled data is presented. In the control group five nontreated CWB males were used. All the mice received $^{125}$I-labeled γ-globulin of Ig-1a type RPC-5 myeloma protein, 1 μg in 0.5 ml of Hanks' balanced salt solution per mouse, intraperitoneally ($1 \times 10^6$ counts per minute per mouse). They were then bled once a day into 5-μl capillary tubes and the radioactivity of the whole blood determined in a scintillation counter. The counts at day 1 were taken as 100% and the counts on successive days compared to this value. The differences between groups were not significant on day 1. The values for individual mice were averaged separately in experimental and control groups. The results are represented in Figure 2. The differences between the results in experimental and control groups are not significant and there is no sign of immune elimination of the labeled γ-globulin antigen. Therefore, if antibodies against donor-type γ-globulin are present in the recipient, their level is probably too low to be an explanation for the elimination of the donor type γ-globulin from the circulation of the host.

To test the possibility of immune rejection of the transferred cells themselves the effect of pretreatment with donor tissues was investigated.

*Cell transfer after presensitization.* Three different experiments were performed. In the first experiment seven CWB females were injected with CSW thymocytes ($2.5 \times 10^6$ thymocytes per mouse, intraperitoneally). Twenty days later these mice were irradiated (300R) and the following day injected with CSW spleen cells ($1 \times 10^6$ cells per mouse, intraperitoneally). In the

second experiment five CWB females were irradiated (300R) and the following day injected with CSW spleen cells ($1 \times 10^4$ cells per mouse, intraperitoneally). As before, the donor γ-globulin increased and subsided. After the disappearance of the donor-type γ-globulin from their circulation (about 5 months after the first irradiation) the recipients were irradiated again with 300R and again injected with CSW spleen cells ($1 \times 10^6$ cells per mouse, intraperitoneally). In the third experiment six CWB females bearing CSW skin grafts were irradiated (300R) and the following day injected with CSW spleen cells ($1 \times 10^6$ cells per mouse, intraperitoneally). In the control experiment, nine CWB females were irradiated (300R) and injected with CSW spleen cells ($1 \times 10^6$ cells per mouse, intraperitoneally).

In all four groups the sera from the recipient mice were collected and tested for the amount of donor-type γ-globulin by the inhibition assay. The results are presented in Figure 3. There is no significant difference in the donor-type γ-globulin level between the control group mice and mice bearing CSW skin grafts. On the other hand, in both groups of CWB mice which were pretreated with CSW spleen or thymic cells, the donor-type γ-globulin level is significantly lower than in the control group. In these cases the recipients act as though they were preimmunized against an antigen(s) present on thymocytes and spleen cells. The possibility of tolerance induction against these cellular antigens was therefore investigated in the next experiment.

*Transfer of different doses of cells.* In this experiment 30 CWB females were

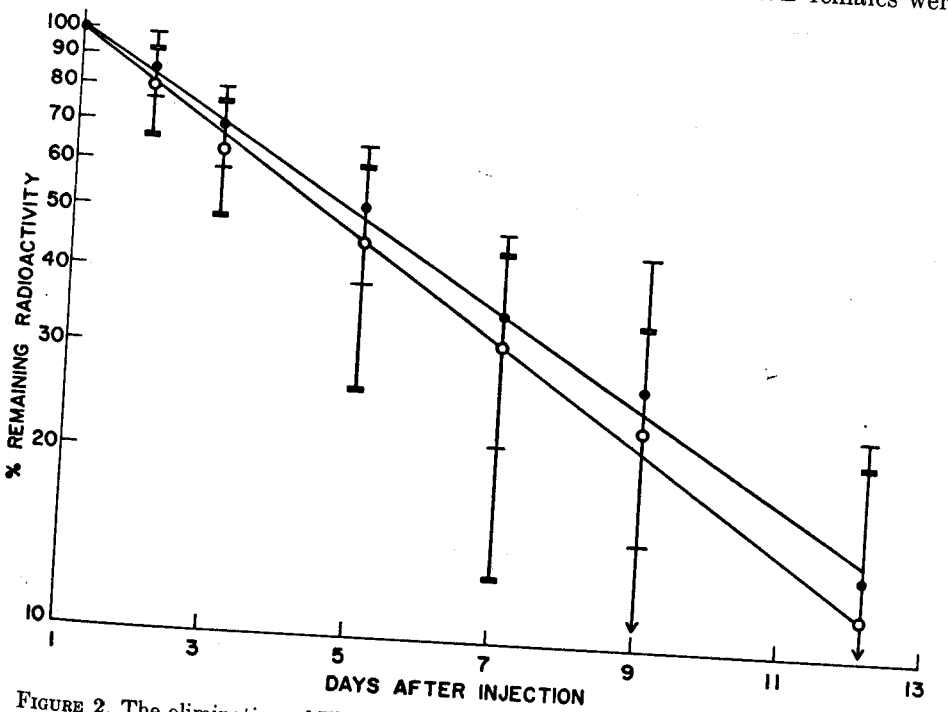


FIGURE 2. The elimination of $I^{125}$-labeled RPC-5 myeloma protein in CWB recipients. ●, Control group; ○, mice pretreated with CSW spleen cells injected with labeled protein, after the disappearance of donor-type γ-globulin.

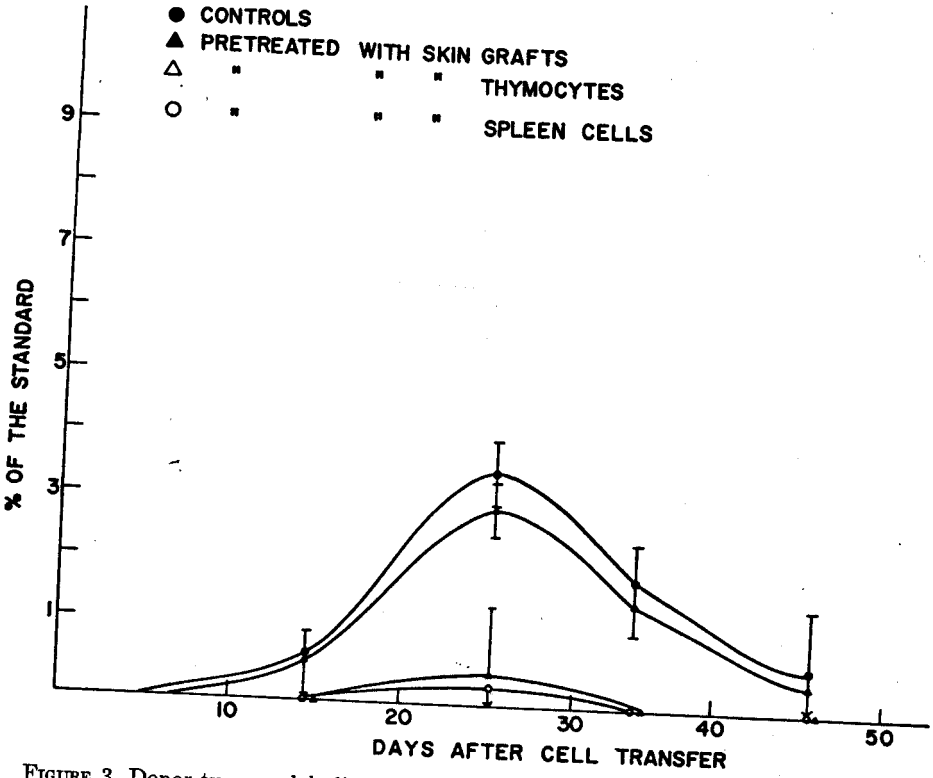


FIGURE 3. Donor-type γ-globulin level in CWB mice irradiated with 300R, pretreated with CSW tissues, and then injected with CSW spleen cells ($1 \times 10^6$ cells per mouse).

irradiated with 300R and then divided into six groups, five animals in each. The following day animals in different groups were injected with different doses of CSW spleen cells, e.g., $10^8$, $10^7$, $10^6$, $10^5$, $10^4$, $10^3$. Sera from these animals were collected and tested for the presence of donor-type γ-globulin. The results are in Figure 4. In all groups, except that injected with the highest dose of cells, the peak of the donor-type γ-globulin level is achieved at about the same time (about 25 days after the cell transfer) and slowly decreases thereafter. At that time there is no significant difference in the donor-type γ-globulin level between the groups injected with $10^6$ to $10^3$ cells. In the group of animals injected with $10^8$ spleen cells the donor-type γ-globulin level continued to rise, finally achieved the level of γ-globulin in standard serum and did not decrease during the observation period. The recipients therefore act as though tolerant to $10^8$ transferred cells.

*The level of host type γ-globulin during the cell transfer experiment.* Two groups of animals from the previous experiment were examined for the presence of host-type γ-globulin—those injected with $10^8$ and $10^7$ spleen cells. In this case, $^{125}$I-labeled C57B1/6 γ-globulin fraction was used as reference antigen in the inhibition assay (with BALB/c anti-B10 allotypic antiserum). The results are represented by the two curves in Figure 5. In the group of animals

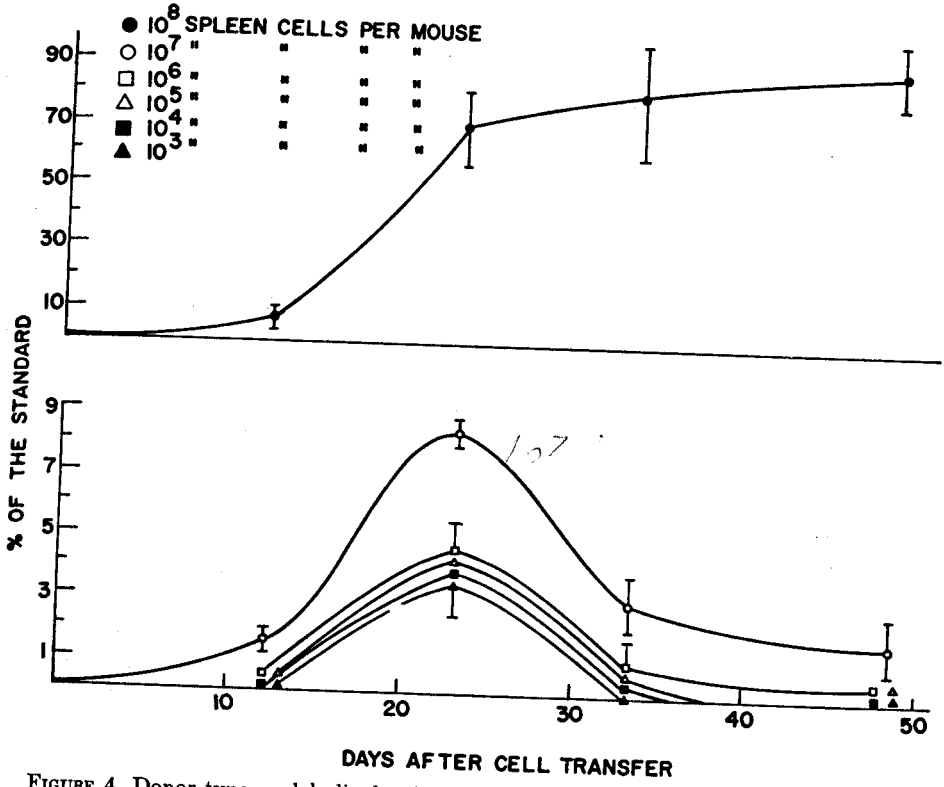


FIGURE 4. Donor-type γ-globulin level in CWB mice irradiated with 300R and injected intraperitoneally with different numbers of CSW spleen cells.

injected with $10^7$ spleen cells the change in the host-type γ-globulin level is not significant. But in the group injected with $10^8$ cells the change is very drastic: The host-type γ-globulin eventually decreases to almost zero level. Comparison of this curve with the corresponding curve in Figure 4 for donor-type γ-globulin shows that as the donor-type γ-globulin level increases, the host-type decreases.

*The γ-globulin production by cells from different tissues.* Twenty-five CWB males and females were irradiated with 600R and then divided into five groups (five animals per group). The following days the groups were injected with CSW cells: group 1, $1 \times 10^6$ spleen cells, intraperitoneally; group 2, $2 \times 10^6$ bone marrow cells intraperitoneally; group 3, $2 \times 10^6$ bone marrow cells intravenously; group 4, $1 \times 10^6$ thymocytes intraperitoneally; and group 5, $20 \times 10^6$ fetal liver cells intraperitoneally (liver from fetuses 17 to 19 days old). During the experiment some of the animals died and at the end of the experiment the numbers of animals in individual groups were 3, 2, 2, 5, and 4 respectively. The results of the experiment are represented by the curves in Figure 6. A detectable level of γ-globulin was found to be produced by spleen cells, bone marrow cells and fetal liver cells, but not by thymocytes. Intravenous injection of bone marrow cells gave a better response than intraperi-

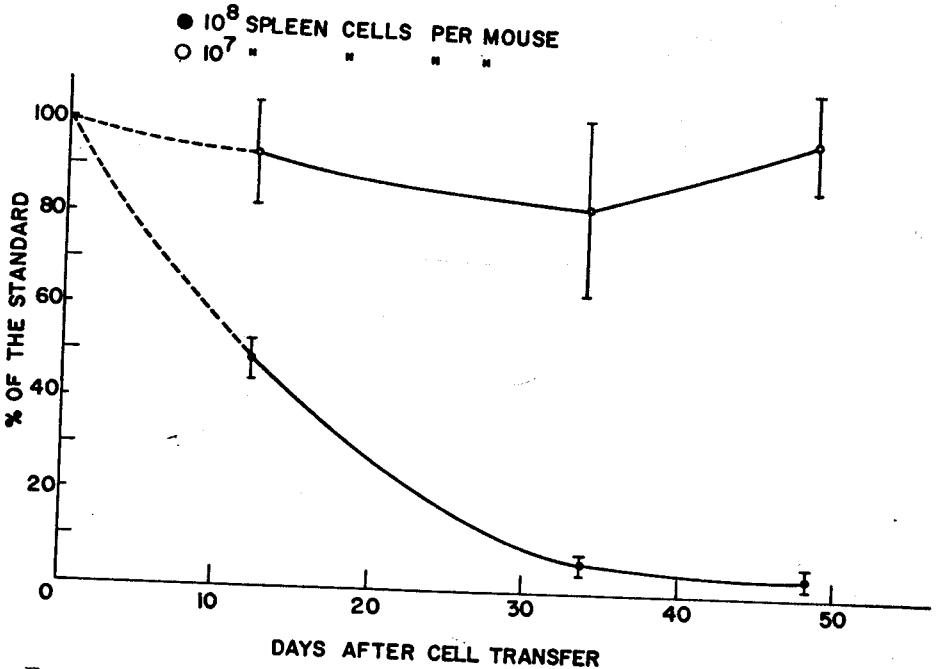


FIGURE 5. Host-type γ-globulin level in CWB mice irradiated with 300R and injected intraperitoneally with CSW spleen cells.

toneal injection. There was no significant difference in the level of γ-globulin produced between spleen cells injected intraperitoneally and bone marrow cells injected intravenously. The γ-globulin production by fetal liver cells was much delayed and by 100 days still much lower compared to spleen cells or bone marrow cells from adult animals.

## DISCUSSION

The data presented indicate that in the CWB/5-CSW congenic pair: (1) there is complete skin histocompatibility, (2) donor-type γ-globulin in more than trace amounts is produced after cell transfer if the recipients have received 300R (or more) X-irradiation, (3) there is an incompatibility for spleen cells which can be overcome by higher irradiation doses or a large number of transferred cells, and (4) sensitization of the recipient against the transferred donor cells can be achieved under appropriate conditions.

These results can be explained as due to two factors: biological space and residual histoincompatibility. The meaning of "biological space" is very vague, but basically it means that the donor cells transferred into unirradiated recipients find themselves at some sort of disadvantage in competition with the host's own cells and are therefore eliminated (or do not become established). Irradiation of the recipients destroy many of their dividing cells and opens an ecological niche for the inoculated donor cells. The higher the dose of irradiation, the more space is made and the more inoculated cells can start functioning and the more donor-type γ-globulin is produced. This space factor plays an important role in cell transfers even in syngeneic combination (*5, 11*).

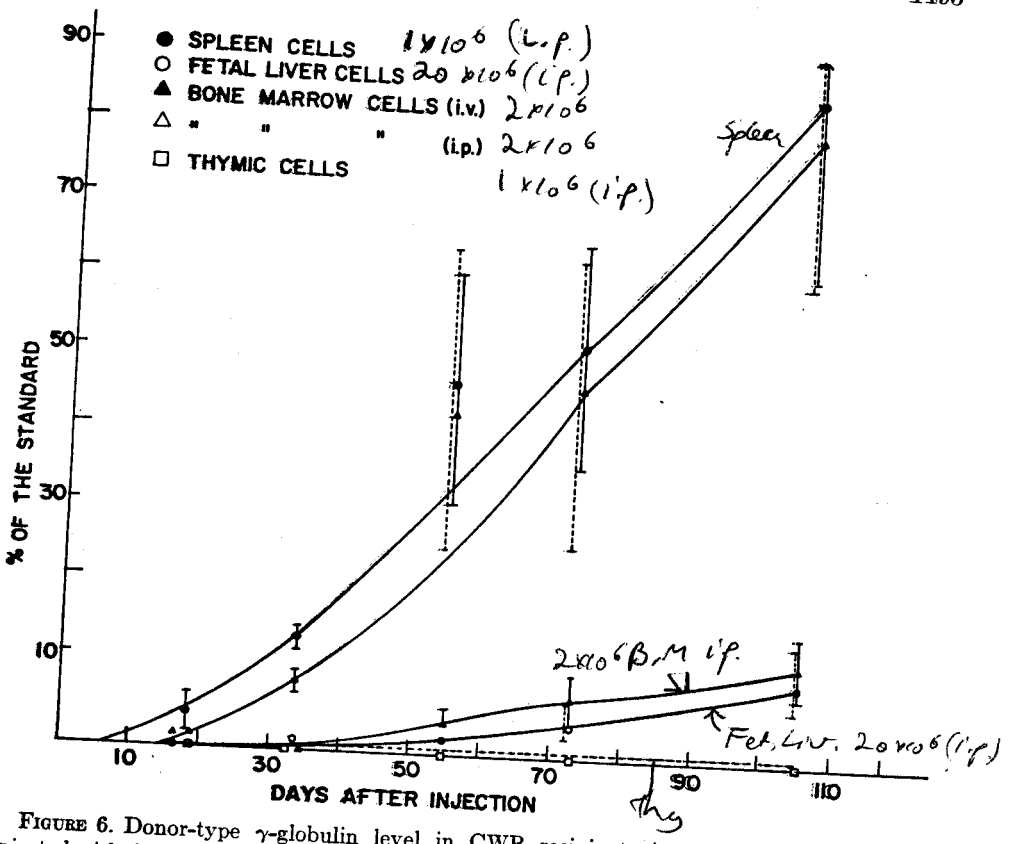


FIGURE 6. Donor-type γ-globulin level in CWB recipients irradiated with 600R and injected with CSW cells from different tissues.

But the space factor does not explain why donor cells which are already established in the recipient discontinue their normal function (production of γ-globulin) after a certain period of time. This cessation of γ-globulin production cannot be caused by depletion of stem cells in the inoculum because in recipients irradiated with more than 300R such a cessation is not observed. The simplest explanation for the cessation is that the transferred cells are rejected by the recipient because of residual histoincompatibility. We must assume in this case, that the CWB/5-CSW congenic pair differs in H-gene(s) which is (are) expressed on spleen cells. It is known that the expression of histocompatibility loci on cells of different tissues is not uniform. Thus, H-2 antigens are present in relatively great amounts on cells of lymphoid tissues but in negligible amounts on erythrocytes, muscle cells, or brain cells (*2*, *8*); on the other hand, antigens determined at the *H-6* locus are primarily present on erythrocytes and brain cells (*1*). It is therefore no surprise that spleen cells have some histocompatibility antigens which skin cells completely lack.

The evidence for residual histoincompatibility is as follows: First, it is possible to achieve a second-set like reaction in this system; a second inoculum of transferred cells, injected after disappearance of γ-globulin produced by the first inoculum has a lowered capability of γ-globulin production. In other

words, the recipient is immunized by the first transferred cells against the second inoculum. Perhaps a direct test for cell survival using radioactively labeled cells would have been useful. The possibility, that the recipient is, in fact, not preimmunized against cellular antigens, but against the γ-globulin produced by the cells, was made unlikely by three factors: (1) the absence of serologically detectable anti-γ-globulin antibodies in the recipients, (2) the nonimmune elimination of passively transferred $^{125}$I-labeled donor γ-globulin, and (3) the finding that thymocytes which, although incapable of producing a detectable level of γ-globulin, sensitize against γ-globulin production by transferred spleen cells. A direct test for cell survival using radioactively labeled cells was not carried out.

Second, the incompatibility between the two congenic lines is sensitive to X-irradiation. The influence of ionizing radiation on transplantation immunity is well established. Very recently, such an influence was thoroughly studied by Brent and Medawar (*4*) who found that whole-body irradiation prolongs the survival of A-strain skin grafts on CBA mice and that over the range 0 to 600R the reciprocal of the survival time is proportional to the applied radiation dosage. Increasing survival time of transferred spleen cells, as judged by the level of γ-globulin produced, with the increased dose of irradiation was found also in our system. At a dose of 600R the recipients became tolerant to the transferred cells. Third, with a high enough dose of transferred spleen cells it is possible to induce a tolerance-like state in recipients treated with an irradiation dose, which allows rejection with a smaller number of transferred cells. The presumptive histocompatibility antigen(s) was proven present on spleen cells and thymocytes and absent from skin. We have no information about its presence on cells from other tissues.

The situation described here is just the opposite of that described by Warner et al. (*17*) in allogeneic mouse radiation chimeras where some recipients rejected skin allografts but had donor-type γ-globulin levels not different from mice with permanently accepted skin grafts. One proposed explanation made by these authors was that some histocompatibility antigen is expressed more strongly in skin cells than in spleen cells.

In the combination CSW-donor and CWB/5-recipient we have not demonstrated antibodies against the γ-globulin produced by transferred cells. Low levels of such antibodies have been demonstrated in the opposite combination (CWB-donor, CSW-recipient (unpublished data)). This difference may be due to the more strongly immunogenic allotypic antigens in the latter case. Although this has not been documented rigorously, we have found it considerably easier to produce antibodies against Ig-1b than against Ig-1a. Nevertheless, an involvement, if any, of these allotypic antibodies in the host-donor cell relationships has not been found.

It should be stressed that donor and host types of only one class ($\gamma G_{2a}$) of immunoglobulins have been followed in these experiments. Whether what happens to this class is an adequate indication of what happens to the other classes remains to be determined.

It was noted that when high levels of donor γ-globulins were present host

type γ-globulins tended to disappear. However, no runting or other overt evidence of graft-versus-host reactions were seen. Whether this is evidence for a mild graft-versus-host reaction or for a kind of allogeneic inhibition, we can not say.

Some of the uses of the congenic immunoglobulin lines is indicated by these studies. Transfers of genetically "tagged" lymphoid cells can be made with sublethal irradiation doses without the consequences of severe graft-versus-host reactions. The production of γ-globulins by a population resulting from an inoculum of as few as a thousand cells can be seen. The smallest inoculum whose product is detectable has not yet been determined. These congenic strains will have a complementary usefulness to the T6-marked congenic lines developed by Lyon (7) since the continued presence and function of the transferred cells can be followed without sacrificing the animal and they do not depend upon cells being in mitosis at the time of examination to be scored as host or donor.

Further backcrossing of this and another congenic line is continuing and it will be of interest to see if the histoincompatibility factor(s) are eliminated by the closer approach to coisogenicity which should thus be obtained.

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