Further Studies on Serum Protein Formation by Chimeras: Induction of Chimerism with a Chemical Carcinogen

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SUMMARY

Rat-into-mouse chimeras were produced by the intravenous injection of rat bone marrow cells after pretreatment of the host at 6 to 10 weeks of age with 3-methylcholanthrene with or without sublethal irradiation. The serum proteins of these chimeras and their sites of production were studied with extensively absorbed antisera specific for rat or mouse serum proteins. Incorporation of \(^{14}\)C-labeled amino acid into serum proteins identified by autoradiography of immunoelectrophoretic patterns from tissue culture fluids from the hosts was used to demonstrate synthesis \textit{in vitro}.

The majority of animals pretreated with 3-methylcholanthrene, with or without sublethal irradiation, were chimeras. Although rat serum proteins never appeared in the serum, rat transferrin and IgG were synthesized \textit{in vitro} by the spleen and mesenteric lymph nodes obtained from these chimeras. Evidence of synthesis of rat serum proteins \textit{in vitro} was found as early as 1 week after bone marrow transplantation. Mouse immunoglobulin production by host spleen and lymph nodes was observed at 2 and 3 weeks after grafting.

INTRODUCTION

It has been shown that lethally X-irradiated mice protected with xenogeneic or allogeneic bone marrow grafts produce donor-type serum proteins in their sera. Lymphoid tissues from such chimeras synthesize donor-type serum proteins \textit{in vitro}. This was demonstrated by the incorporation of \(^{14}\)C-labeled amino acids into serum proteins by tissues \textit{in vitro} with the technique of autoradiography of immunoelectrophoretic patterns of concentrated culture fluids obtained from these tissues (13, 14).

Recently, the efficacy of the antineoplastic agent, cyclophosphamide, in the induction of chimerism in BALB/c mice given an intravenous inoculation of allogeneic spleen cells (4) was reported. With the same agent it was shown that temporary xenogeneic chimerism (rat-into-mouse) involving the hematopoietic cells could be produced allowing the hosts to synthesize the rat hemoglobin that forms the haptoglobin-hemoglobin complex (12). Although the mechanism of action is not entirely clear, carcinogenic hydrocarbons such as methylcholanthrene and dimethylbenzanthracene have been shown to depress the homograft reaction (9, 19, 21).

The experiments reported in this paper were undertaken to determine whether the depression of the immune response in MCA-treated mice would be sufficient to permit the survival of foreign cells, cells of xenogeneic origin, in such hosts by using donor-type serum protein production by host tissues \textit{in vitro} as a marker.

MATERIALS AND METHODS

Production of Chimerism. C57BL/6 male mice (Jackson Memorial Laboratory, Bar Harbor, Maine) weighing 25 to 40 g were used as the recipients of bone marrow cells obtained from Sprague-Dawley male rats weighing 200 to 250 g (Blue Spruce Farms, Altamont, N. Y.). The cell suspensions were collected in Medium 199 (Microbiological Associates, Bethesda, Md.) from tibiae, femora, and humeri. The cells were washed once, filtered through layered gauze, and adjusted to the desired concentration. The recipients, which were divided into 4 groups, were given intravenous injections of 8 to 15 x 10\(^7\) bone marrow cells in a volume of 0.5 ml each after the induction of chimerism by one of the following methods: Group 1, 5 mg 3-methylcholanthrene (Eastman Organic Chemicals, Rochester, N. Y.) and 450 R whole-body X-irradiation; Group 2, 5 mg MCA alone; Group 3, 10 mg MCA and 450 R whole-body X-irradiation; Group 4, 10 mg MCA alone.

There were 14 mice in each group. In each group 8 animals received their bone marrow injection 1 week after the initial procedure; the others received it 2 weeks later. Subcutaneous inoculations of MCA were given bilaterally in divided doses in the axillary and femoral regions. In the groups in which the animals were irradiated, the bone marrow cells were given within 4 hr of the X-irradiation. The conditions of the X-irradiation have been described previously (13).

After the injections of MCA, the mice received Terramycin in their drinking water and Aureomycin (Aurofac, General Bio-

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The abbreviations used are: MCA, methylcholanthrene; Tr, transferrin.
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chemicals, Chagrin Falls, Ohio), in their food, approximately 6 mg/kg/day.

Blood samples for immuno-electrophoretic analysis of sera and alkaline phosphatase reaction of peripheral leukocytes for identification of rat cells in the circulation were obtained from the retroorbital plexus of individual animals at weekly or biweekly intervals. Sera were stored at -20° until analyzed by microimmuno-electrophoresis according to the method of Scheidegger (20).

Blood smears were stained by the method of Kaplow (8) for leukocyte phosphatase activity of blood and bone marrow. Quantitative determinations of peripheral leukocytes were made every week with a standard hemocytometer.

Cultures and Analysis of Culture Fluids. Mice from each group were sacrificed weekly and their spleens and mesenteric lymph nodes were removed aseptically. Approximately 80 to 100 mg spleen and 50 to 70 mg pooled mesenteric lymph nodes were minced and cultured for 24 to 48 hr at 37° in roller tubes with 1 to 2 ml medium as previously described (6). 14C-Labeled lysine and isoleucine, 100 to 200 µCi/mmol (Schwarz BioResearch, Orangeburg, N. Y.), were added to a concentration of 1 µCi/ml each.

After incubation the culture fluids were dialyzed against 0.015 M phosphate buffer at pH 7.2 for 48 hr, lyophilized, and redissolved with 0.15 ml distilled water.

Identification of the labeled rat proteins in the concentrated culture fluids was carried out with an unlabeled rat carrier serum which was added to the antigen well prior to the addition of the concentrated culture fluids in order to provide enough rat proteins to develop immunoelectrophoretic patterns. These patterns were developed with goat antirat sera (Hyland Laboratories Inc., Los Angeles, Calif.) which was extensively absorbed with lyophilized mouse tissues (liver and spleen), lyophilized mouse serum, and fresh mouse serum. Any 14C-labeled rat serum proteins present in the culture fluids were precipitated by the antiserum together with carrier serum proteins in the corresponding immuno-electrophoretic lines and demonstrated by autoradiography of the washed and dried slides, as previously described (7).

For demonstration of the production of mouse serum proteins, labeled mouse serum proteins were similarly identified by the use of a mouse serum carrier and rabbit antirat serum (Immunology, Inc., Glen Ellyn, Ill.) absorbed with rat tissues and serum.

Identification of the immunoelectrophoretic arc of transferrin was made by means of 59FeCl3. Sera or culture fluids were incubated for 1 hr at 37° with 2 µCi 59FeCl3/ml. Autoradiographs of the immunoelectrophoretic patterns of these sera or culture fluids were prepared with Kodak Royal Pan sheet film, exposure time 2 weeks.

RESULTS

The mean survival time cannot be accurately stated because the number of animals in each group was small and the animals were sacrificed at intervals for organ cultures. In general the animals receiving MCA without sublethal irradiation (Groups 2 and 4) followed by bone marrow transplantation 1 week after MCA injection survived longer than animals in the groups receiving the bone marrow grafts 2 weeks after MCA injection (Table 1). Histological sections of some of the spleens from an occasional animal in these groups revealed severe atrophy of the white and red pulp with diffuse fibrosis and hemosiderin deposition.

The majority of the survivors were chimeras as demonstrated by the incorporation of 14C-labeled amino acids into donor-type γ-globulin (Table 2). Culture fluids from spleen and peritoneal macrophages from Strain A mice did not show

| Table 1 |

| General features of chimeras after various methods of induction |

<table>
<thead>
<tr>
<th>Method of induction of chimerism</th>
<th>Time of bone marrow grafting (wk)</th>
<th>No. of animals</th>
<th>Survival time</th>
<th>Spleen size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(of majority of animals)</td>
</tr>
<tr>
<td>MCA (mg) X-ray</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 + X-ray</td>
<td>1</td>
<td>8</td>
<td>Several deaths during the 3rd and 4th wk (other animals used for cultures)</td>
<td>Enlarged</td>
</tr>
<tr>
<td>5 - X-ray</td>
<td>1</td>
<td>8</td>
<td>Most animals survived 5 wk; 1 to 10 wk, bearing an MCA-induced tumor</td>
<td>Enlarged</td>
</tr>
<tr>
<td>5 - +</td>
<td>2</td>
<td>6</td>
<td>No survivals after 3 wk</td>
<td>Small</td>
</tr>
<tr>
<td>10 + X-ray</td>
<td>1</td>
<td>8</td>
<td>Deaths usually at 2 to 4 wk</td>
<td>Small</td>
</tr>
<tr>
<td>10 - X-ray</td>
<td>1</td>
<td>8</td>
<td>Some survivals at 5 wk with 1 survivor until 14 wk with an MCA-induced tumor</td>
<td>Average or enlarged</td>
</tr>
<tr>
<td>10 + X-ray</td>
<td>2</td>
<td>6</td>
<td>No survivors after 3 wk</td>
<td>Small</td>
</tr>
<tr>
<td>10 - X-ray</td>
<td>2</td>
<td>6</td>
<td>No survivors after 3 wk</td>
<td>Small</td>
</tr>
</tbody>
</table>

aNo. of weeks after MCA inoculation.

bNo. of weeks after bone marrow transplantation.
Table 2

<table>
<thead>
<tr>
<th>Tissues for culture</th>
<th>Week after bone marrow transfer</th>
<th>Donor-type γ2-globulin synthesis*</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
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<td>Spleen (individual)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>2</td>
<td>0 0 0 2 2 0 1 1 1 0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>3</td>
<td>0 0 0 0 2 2 0 1 1 1</td>
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<td>+</td>
<td>+</td>
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<tr>
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<td>4</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>5</td>
<td>0 0 0 0 0 0 1 1 1 0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Mesenteric lymph nodes (pooled from 2 animals)</td>
<td>1</td>
<td>0 0 0 0 0 1 1 1 1 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

*Group 1, 5 mg MCA and sublethal irradiation; Group 2, 5 mg MCA alone; Group 3, 10 mg MCA and sublethal irradiation; Group 4, 10 mg MCA alone.

Each figure represents the number of cultures in which rat IgG2 was found labeled.

The labeling of rat serum proteins when developed with a rat carrier and absorbed antirat serum. Culture fluids obtained from the spleens of chimeric animals initially receiving 5 mg MCA and X-irradiation (Group 1) showed weak but definite rat IgG2 synthesis at 2, 3, and 4 weeks after bone marrow injection. In the 5-mg, MCA-nonirradiated group of animals (Group 2), strong rat IgG2 synthesis could be demonstrated at 1, 3, and 5 weeks after bone marrow injection (Fig. 1).

The mesenteric lymph nodes also synthesized rat IgG2 globulin, although the intensity and frequency of appearance of the labeling were less than with spleen cultures from the same animals.

The mice that received 10 mg MCA prior to sublethal irradiation (Group 3) did not survive more than 3 weeks. Spleen cultures were obtained only at 3 weeks in this group and showed weak rat IgG2 synthesis. Because the mortality was very high, cultures were obtained at earlier intervals from some of the animals in this group that received bone marrow injections 2 weeks after the initial procedure. There was no evidence of synthesis of rat immune globulins by lymphoid tissues in vitro before 3 weeks.

Culture fluids from spleens and lymph nodes of mice receiving 10 mg MCA without X-irradiation and bone marrow grafts (Group 4) 1 week after MCA showed labeling of rat IgG2 at 3 and 5 weeks. A spleen culture from 1 animal bearing an MCA-induced tumor and surviving 14 weeks after MCA injection did not show synthesis of γ-globulin of donor origin.

The results obtained in all 4 groups when the bone marrow transplant was given 2 weeks after MCA injection (with or without X-irradiation) were not as consistent. Spleen cultures from only an occasional animal showed rat IgG2 synthesis 3 weeks after bone marrow transfer.

Rat proteins were not demonstrated in sera of any of the hosts, including those for which spleen cultures showed γ-globulin production in vitro. Of the 4 serum samples obtained at 2 or 3 weeks from animals in each of the 4 groups and specifically examined for rat Tr, none were positive. Mouse Tr was present in 3 of the 4 sera. Spleen cultures from the same animals at the same intervals showed strong labeling of rat and mouse Tr (Fig. 2).

Mouse immunoglobulin production by spleen tissues from chimeric animals was observed in all groups at 2 and 3 weeks after bone marrow grafting (Table 3). One-week spleen cultures were obtained only from animals in Groups 2 and 4. These showed very strong IgG2 labeling by the spleen, and weak to moderate labeling by the mesenteric lymph nodes (Fig. 3). The spleens from chimeric animals in Groups 1 and 2 showed the strongest mouse immune globulin synthesis at the other intervals studied. The mesenteric lymph nodes from animals in all 4 groups showed less synthesis of mouse immune globulins at the same intervals than did their spleens.

Table 3

<table>
<thead>
<tr>
<th>Tissues for culture</th>
<th>Mouse γ2-globulin synthesis*</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
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<tbody>
<tr>
<td>Spleen (individual)</td>
<td>1 2 0 0 1 1 1 0 1 1 1 1 1 1 1 1</td>
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<td>+</td>
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<td>+</td>
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<tr>
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<td>5</td>
<td>0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mesenteric lymph nodes (pooled from 2 animals)</td>
<td>1</td>
<td>1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1</td>
<td>+</td>
<td>+</td>
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<td></td>
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<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
<td>+</td>
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<tr>
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<td>3</td>
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<td>+</td>
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<tr>
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<td>4</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
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<td>+</td>
<td>+</td>
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<td>14</td>
<td>1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Treatment of groups is described in Table 2, Footnote a.

Each figure represents the number of cultures in which rat IgG2 was found labeled.

Staining for alkaline phosphatase activity of peripheral blood cells from treated mice was carried out weekly. However, no positive cells were found at any interval over a 5-week period of observation after bone marrow transfer, even in mice in which spleen cultures showed synthesis of rat γ-globulin.

The numbers of peripheral leukocytes were reduced throughout the 5 weeks after MCA (5 or 10 mg) administration. The leukocyte counts of the animals that received MCA (5 or 10 mg) and sublethal irradiation were lower than those of animals receiving MCA alone. The period of lowest cell count was obtained 3 or 4 weeks after MCA injection (Table 4).

DISCUSSION

There is a large body of literature relating to the carcinogenic action of polycyclic hydrocarbons, particularly MCA. This effect is generally held to be a direct transforming action of
These difficulties were overcome to some extent by the enhancement phenomenon. Production and that it is perhaps similar generically to the cellular immune process rather than on humoral antibody recipients as compared with untreated controls (18). It was suggested that the prolonged graft survival depends on the function of the graft rejection mechanism that is the sole effect, but it may influence the effectiveness of some carcinogens as tumor inducers.

The earlier quantitative studies of tumor growth enhancement in carcinogen-treated hosts were found to be limited by the early death of the host as well as "antineoplastic" effects of the substances that were used to promote graft survival. These difficulties were overcome to some extent by the transplantation of normal tissue, usually skin. Prolonged allogeneic graft survival was attainable in carcinogen-treated recipients as compared with untreated controls (18). It was suggested that the prolonged graft survival depends on the cellular immune process rather than on humoral antibody production and that it is perhaps similar generically to the enhancement phenomenon.

It has already been shown that MCA promotes tumor induction and allograft survival. The present studies provide evidence for xenograft survival. So that the immune response could be depressed sufficiently for the establishment of a xenogeneic system, the dosage of MCA in these studies could be depressed sufficiently for the establishment of a xenogeneic system, the dosage of MCA in these studies exceeded the ordinary carcinogenic doses. It is conceivable that the dosages used caused the intensive reduction of bone marrow cells, lymphoid cells, and peripheral blood cells of both host and donor origin. This could account for the marked leukopenia and the fact that alkaline phosphatase-positive cells could not be found in the peripheral blood of chimeric animals.

The fact that the mice that received rat bone marrow transplants 1 week after MCA injection survived longer than those mice receiving their grafts at 2 weeks may indicate the marked suppressive effect of MCA on the immunocompetent cells of the host with subsequent development of the graft-versus-host reaction as opposed to the earlier protective effect of the rat bone marrow when given at 1 week. The failure to detect rat serum protein synthesis in the sera of these chimeric mice as compared with those induced by lethal X-irradiation may be due to a diminished synthesis of rat proteins by MCA-induced xenogeneic chimeras and that in contrast to X-irradiation, the toxic effect of MCA on the donor cells is still present.

Experiments in this laboratory establish the fact that donor-type serum proteins are formed in vitro by lymphoid tissues from MCA-induced rat-into-mouse chimeras with or without sublethal irradiation. In the past chromosome markers (1) and cytotoxic assay (2) have been used as indicators of the establishment of successful chimerism. More recently, in vitro production of donor type serum proteins by lymphoid tissues from postirradiation xenogeneic and allogeneic chimeras (5, 6) has been used as a marker. Previous experiments in this laboratory have shown that in postirradiation xenogeneic chimeras, donor-type γ-globulin first appeared in the serum 4 weeks after bone marrow transplantation and gradually increased over a 14-week interval. Donor type immune globulin production in vitro by the spleens of the same chimeric animals first appeared at 4 weeks, while mouse immune globulin production was seen only by the lymphoid cells of those chimeras surviving 2 to 3 months. In the present experiments labeling of IgG2 of both donor and host origin from the outset was present throughout the duration of the experiments. These findings are similar to those reported recently with cyclophosphamide-induced rat-into-mouse chimeras (12) in which labeling of mouse Tr and immune globulins was seen in the first and second week respectively although culture fluids obtained from the spleens of these animals did not show any labeled rat serum proteins during the 9-week period of observation. The fact that these latter animals were chimeras for several weeks after rat bone marrow injection was established by the presence of rat hemoglobin forming the haptoglobin-hemoglobin complex in their sera. These results are also in agreement with the findings in neonatal chimeras induced with rat spleen or thymus cells (15) in which rat immune globulin formation appeared early without evidence of production of any other rat proteins. Graft-versus-host reactions were produced by both spleen and thymus and the variety and amount of donor-type serum proteins observed in the sera of these neonatal chimeras was much less than that described in post-X-irradiation chimeras.

The labeling of both rat and mouse immunoglobulin by chimeric tissue in vitro almost from the outset in the present study suggests that the mechanism of action of MCA in prolonging graft survival is not to abolish totally the capacity for γ-globulin synthesis.

The mechanism by which MCA depresses homograft reactivity is as unknown as the mechanisms by which radiation and other cytotoxic agents do so. However, the oncogenic, mutagenic, and toxic effects of chemical carcinogens are comparable with those produced by radiation. Interactions between carcinogens, nucleic acids, and proteins suggest that interference with biosynthetic mechanisms comparable to that produced by radiation and cytotoxic agents is involved.

**ACKNOWLEDGMENTS**

The excellent technical assistance of Mrs. Yvonne Simmons is gratefully acknowledged.
REFERENCES


Fig. 1. Autoradiograph (AR) of immunoelectrophoretic pattern (IE) obtained with a spleen culture obtained 1 week after bone marrow transplantation. The pattern was developed with rat serum (RS) as a carrier and rabbit antirat sera absorbed with mouse tissues and serum. Chimerism was induced with 5 mg MCA without sublethal irradiation. There is strong labeling of IgG2 (72) and IgG1 (71).

Fig. 2. Autoradiograph (AR) of immunoelectrophoretic pattern obtained with a spleen culture fluid obtained from a 5-mg MCA (without sublethal irradiation) chimeric animal 2 weeks after bone marrow transplantation. Both rat and mouse transferrin (7>) are present. Mouse and rat carrier sera and absorbed antimouse sera were used to develop the respective immunoelectrophoretic patterns.

Fig. 3. Autoradiograph (AR) of immunoelectrophoretic pattern (IE) obtained with spleen culture from chimeric animals receiving 5 mg MCA, without X-irradiation. The patterns were developed with mouse serum as a carrier and absorbed antimouse sera. There is strong mouse immunoglobulin labeling.
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