Immunogenicity and Partial Purification of Soluble H-2 Antigens Extracted with Hypertonic Salt

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IMMUNOGENICITY AND PARTIAL PURIFICATION OF SOLUBLE H-2 ANTIGENS EXTRACTED WITH HYPERTONIC SALT

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H-2^d antigen was solubilized by 3 M KCl extraction from cultured murine lymphoid cells L1210, derived from DBA/2 mice. Yields of protein and specific inhibitory activity were found to be optimal when the cells were harvested during log growth. A 5- to 7-fold increase in specific H-2^d antigen activity was achieved by Sephadex gel filtration and cellulose ion exchange chromatography. Antibodies against H-2K^d and H-2D^d determinants were produced by immunization experiments within the allogeneic system. Immunization with soluble H-2^d antigen led to specific sensitization with respect to cellular immunity, indicating that the soluble material maintained its integrity as a serologically defined histocompatibility antigen.

Ever since Medawar (1) observed that solubilized cell surface antigens administered i.v. tend to prolong graft survival, many efforts were made to elucidate the chemical nature of these transplantation antigens in order to gain a better understanding of cell membrane structure and function and to clarify the role of these antigens in cell regulatory mechanisms and the immune response (2-10). Thus, a variety of methods were developed in the last decade to solubilize transplantation antigens from their sites on the surfaces of cell membranes (11, 12).

In the present study H-2 antigens were solubilized with 3 M potassium chloride, a method already found highly suitable for the solubilization of HL-A antigens (13). Our objectives were: 1) to evaluate the immunologic potency of solubilized, serologically active H-2 antigens in vivo by determining their ability to provoke specific humoral and cellular immune responses, and 2) to assess whether partial purification would result in increased antigenicity detectable by serologic analyses.

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MATERIALS AND METHODS

Animals. The following strains of mice were used for immunization and transplantation experiments: B10.D2 new/Sn (H-2^a) and C57BL/10Sn (B10) (H-2^b). For transplantation experiments only female mice were utilized ranging in age from 7 to 15 weeks.

For serologic analysis of H-2 determinants the following additional strains of mice were used: B10.A/Sn (H-2^a), B10.M/Sn (H-2^b), B10.A(2R)/SgSn (H-2^12a), B10.A(5R)/SgSn (H-2^12b), B10.BR/SgSn (H-2^k), B10.P (H-2^p), B10.RHI7(IN)/Sn (H-2^r) and B10.S (H-2^s). All these strains were bred onto the background of B10 mice. Additional strains of mice used were as follows: C3H/HeJ (H-2^k), C3H.JK/Sn (H-2^k), C3H.NB/Sm (H-2^k), DBA/2J (H-2^a), HTG (H-2^a), AKR.M (H-2^m) and DBA/1J (H-2^b). The complex H-2 locus is separable into at least the "D" and "K" regions separated by the Ir locus (14, 15) and Ss-Slp trait (16, 17) as proposed by Shreffler et al. (18), Snell et al. (19) and Klein and Shreffler (20). According to a recent H-2 chart (17), the H-2^a allele has H-2 antigens: 4, 3, 6, 13, 27, 28, 29, 35, 36, 41, 42, 43, 44 derived from the D region of the locus and H-2^b antigens: 31, 3, 8, 27, 28, 29, 34, 46 derived from the K region of the locus (the private specificity is italicized).

Mice were obtained from the Jackson Labora-
Solubilization of H-2d antigens. Soluble antigens were extracted by the 3 M KCl method essentially as described previously for the solubilization of HL-A antigens (13). The antigen source material consisted of cultured murine cells L1210, derived from a lymphoid tumor in ascites form from DBA/2 (H-2d) mice. The antigen preparation is therefore called H-2d antigen. The cells were maintained in continuous culture in medium RPMI 1640 + 10% fetal calf serum (FCS). The cultured cells had a doubling time of 11 to 13 hr at 36.5°C and were harvested for antigen extraction during their log growth phase. L1210 cells grown as ascites were obtained 5 days after inoculating 2 × 10⁷ cultured L1210 cells into B10.D2 mice. Spleen cells were obtained by teasing apart the spleen and resuspending the cells in phosphate-buffered saline, pH 7.2. Red cells were removed by incubation of the cell suspension in 0.83% NH₄Cl (10 ml/10⁸ cells) for 5 min at room temperature and then washing them twice with saline.

In a typical experiment, cells were washed twice in cold saline and then adjusted to a concentration of 10⁸ cells per milliliter. Potassium chloride was slowly added to a final concentration of 3 M. The hypertonic cell suspension was incubated for 16 hr with gentle shaking at 4°C. The extract was then ultracentrifuged for 90 min at 104,000 × G and the resultant supernatant diluted 1:10 with distilled water. After centrifugation for 15 min at 10,000 rpm and removal of a precipitate consisting largely of nucleic acids, the extract was concentrated to ~10 mg protein per milliliter with Amicon ultrafiltration chambers (Amicon Corp., Lexington, Mass.) equipped with UM 10 filters.

Gel filtration. Sephadex G-100 (Pharmacia, Uppsala, Sweden) was equilibrated before use with 0.10 M Tris-HCl buffer, pH 7.2. Flow rate of the G-100 column (2.5 x 60 cm) was maintained at 0.5 ml/min at 4°C. The elution profile was recorded by a Uvicord II densitometer at a wavelength of 256 nm.

DEAE-cellulose chromatography. DEAE-cellulose (DE 52, Whatman) was equilibrated with Tris-HCl buffer (0.01 M Tris, 0.05 M KCl), pH 8.1, packed into a column and then eluted stepwise with 0.01 M Tris buffers, pH 8.1, containing increasing amounts of KCl, i.e., 0.1, 0.2, 0.3, and 0.5 M. The elution rate was maintained at 24 ml/hr and 6-ml fractions were collected. The elution profile was determined by automatic recording with a Uvicord II densitometer at a wavelength of 256 nm.

Disc electrophoresis. Discontinuous electrophoresis on 7.5% polyacrylamide gels was performed according to the method of Rodbard and Chrambach (21). For analytical gels, approximately 60 to 120 μg of protein were applied per (0.6 x 10 cm) gel. A constant current of 2.5 mA was applied per gel and after the bromphenol blue marker reached the end of the gel, electrophoresis was discontinued with the gel being removed and stained with 0.1% Coomassie Blue.

Mice (from 12 to 14 in each group) were immunized with H-2d antigen as outlined in Table I. The antigen injected was 3 M KCl extract, ultracentrifuged and concentrated on Amicon filter UM 10. Antigen preparations used for immunization had a serologic activity of at least 8,000 to 12,000 ID₅₀ units for H-2.4 and from 2,500 to 4,000 ID₅₀ units for H-2.31 per milligram of protein. In addition to those animals listed in Table I, some B10 mice were injected only once with either 100 μg or 10 μg antigen subcutaneously and then each received a B10.D2 skin graft 6 days later. As controls, B10 mice each received two B10.D2 skin grafts at a time interval of 4 weeks.

In general, mice were bled 1 day before injection of antigen, 7 and 14 days after each injection, and then at weekly intervals for 4 to 6 weeks after the last challenge with antigen.
**Lymphocytotoxic test.** A modification of the eosine micromethod of Mittal et al. (23) was employed as described previously (24, 25) and lymph node lymphocytes from different mouse strains were utilized as target cells. The lymphocytes were obtained from inguinal, axillary, and mesenteric lymph nodes by teasing the nodes apart and suspending the cells in medium RPMI 1640 + 10% heat-inactivated FCS (iFCS). After removal of tissue and cell debris by low speed centrifugation (1 min at 400 \( \times G \) in an International Centrifuge), the cells were washed twice in medium RPMI 1640 + FCS and finally adjusted to a concentration of 2 \( \times 10^6 \) cells/ml.

Selected normal rabbit serum mixed in a ratio of 1:3 with guinea pig serum served as a source of complement. This mixture was shown previously to possess little nonspecific cytotoxic activity and proved to be highly efficient in the lymphocytotoxic reaction (24).

**The two-stage lymphocytotoxic test.** In this test, target cells together with appropriate dilutions of H-2 alloantisera were incubated at room temperature. After 60 min the cells were washed three times in medium RPMI 1640 + 10% iFCS and adjusted to 10^6 cells/ml. Two microliters of this cell suspension were placed onto microtiter plates (Möller-Coats, Norway) and incubated with 2 \( \mu l \) of complement for 60 min at room temperature. After the cells were stained with 2 \( \mu l \) eosin, the reaction was stopped by adding 2 \( \mu l \) of 36% formalin.

**Cytotoxic inhibition test.** The cytotoxic inhibition test was performed essentially as the lymphocytotoxic test described above. However, before reacting target cells with antiserum, appropriate dilutions of antiserum were incubated with 2-fold progressive dilutions of H-2\(^a\) antigen for 60 min at room temperature. Thereafter the target cells were added, and 30 min later complement was admixed. After another 60-min incubation at room temperature, eosin was added. The reaction was stopped by the addition of formalin.

The results were expressed as \( \text{ID}_{50} \) units, i.e., the amount (micrograms) of antigen required to halve the cytotoxic power of a specific alloantiserum used at a dilution equivalent to cause 80 to 95% cell death (zero cytotoxic units). The \( \text{ID}_{50} \) was calculated as:

\[
\% \text{ Inhibition} = 100 - \frac{\% \text{ cells killed in presence of inhibitor}}{\% \text{ cells killed in absence of inhibitor}} \times 100
\]

**Absorption experiments.** Absorptions were performed by a micromethod (24). Varying amounts of packed cells were incubated with agitation at 10-min intervals for 60 min at room temperature in Beckman microfuge tubes with a constant amount of undiluted antiserum. The tubes were centrifuged for 5 min at 13,000 \( \times G \) in a Beckman microfuge and the supernatant was then transferred to other Beckman tubes. The absorbed sera were stored at -20°C until they were tested for residual cytotoxicity against suitable target cells in the direct lymphocytotoxic test. The results were expressed as \( \text{AD}_{50} \) units (absorbing dose), i.e., the number of cells per microliter which was necessary to halve the cytotoxic activity of 2 \( \mu l \) undiluted antiserum.

**Immunodiffusion analyses.** Double microimmunodiffusion tests were performed by applying 2 ml of 1% agar (Ionagar; Colab Laboratories, Chicago, Ill.) in 0.15 M potassium phosphate buffer, pH 7.0, plus 0.5 M glycine onto each microslide. Samples (7 to 15 \( \mu l \)) were applied, and the reaction was allowed to develop at room temperature for 3 days. Precipitin

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**TABLE I**

<table>
<thead>
<tr>
<th>Regimen</th>
<th>1st Injection of Antigen*</th>
<th>Interval to Booster</th>
<th>Booster Injections*</th>
<th>Interval between Boosters</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>(a) 0.01 i.v.</td>
<td>4 weeks</td>
<td>2 ( \times 0.1 \text{s.c., w/o} )</td>
<td>2 weeks</td>
</tr>
<tr>
<td>B</td>
<td>0.1 w/o</td>
<td>4 weeks</td>
<td>2 ( \times 0.1 \text{s.c., w/o} )</td>
<td>2 weeks</td>
</tr>
<tr>
<td>C</td>
<td>0.1 s.c., CFA</td>
<td>4 weeks</td>
<td>2 ( \times 0.1 \text{s.c., CFA} )</td>
<td>2 weeks</td>
</tr>
<tr>
<td>D</td>
<td>0.1 s.c., CFA</td>
<td>1 week</td>
<td>4 ( \times 0.1 \text{s.c., CFA} )</td>
<td>1 week</td>
</tr>
</tbody>
</table>

* i.v., intravenous; s.c., subcutaneous; w/o, without adjuvant; CFA, complete Freund’s adjuvant.
lines occurred usually after 12 to 16 hr.

Skin grafts. Full thickness circular pinch grafts of 1.1 cm in diameter were used as skin transplants. The corresponding patch removed from the recipient was 1.0 cm in diameter. The grafts were placed on the left back and secured with a bandage. The bandage was changed on days 5, 7, 8, and 9 and removed on day 10 to permit scoring. Grafts were scored as rejected if they were either sloughed, thick, hard, discolored, or shrunken. Occasionally histologic examinations were performed on days 6, 8, 10, and 12 after grafting. Mean survival times were calculated, and differences between groups were tested for statistical significance by the Wilcoxon, Mann-Whitney rank test (26).

RESULTS

Solubilization of H-2 antigens. Table II shows typical activities and yields of H-2a antigens obtained from cultured L1210 cells by hypertonic salt extraction and of L1210 ascites cells harvested 5 days after i.p. injection of $2 \times 10^7$ cultured L1210 cells into B10.D2 mice. When cultured cells L1210 were used, optimal yields (15 mg protein/10^9 cells) and antigenic activities of 0.08 ID_{50} units (H-2.31)/μg protein were obtained when cultures were harvested during mid-log growth. These data were obtained from 22 different extraction experiments. The average yield of antigen from 10^9 cells was 12 mg of protein with average antigenic activity of 0.15 (H-2.4), 0.20 (H-2.28), and 0.12 (H-2.31) ID_{50} units/μg protein, respectively. In late log growth or in resting phase, yields of H-2 antigens were low (27) (Fig. 1). When cultured cells with

![Figure 1. Cytotoxic inhibition of alloanti-H-2 sera by soluble H-2a antigen. The activity of two H-2a antigen preparations is shown: 1) antigen prepared from cultured L1210 cells harvested in mid-log phase (inhibition of anti-H-2.4, A A; anti-H-2.28, △ -- △; anti-H-2.31, O -- O; and anti-H-2.8, ▲ -- ▲) and 2) antigen prepared from cultured L1210 cells harvested in resting phase (inhibition of anti-H-2.4, △ -- △, and anti-H-2.31, □ -- □). The alloantisera were used at the last dilution causing 80% cell death; target cells were L1210 cultured cells.](http://www.jimmunol.org/)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cells × 10^9</th>
<th>Protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Protein Mg/10^9 cells</th>
<th>ID_{50} Units (μg Antigen)</th>
<th>ID_{50} Units × 10^4 per Mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210</td>
<td>8.5</td>
<td>8.9</td>
<td>94.5</td>
<td>11</td>
<td>0.11</td>
<td>0.16</td>
</tr>
<tr>
<td>L1210</td>
<td>9.8</td>
<td>9.8</td>
<td>94.5</td>
<td>20</td>
<td>0.11</td>
<td>0.16</td>
</tr>
<tr>
<td>B10.D2</td>
<td>12</td>
<td>3</td>
<td>78</td>
<td>6.5</td>
<td>0.09</td>
<td>0.07</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>10</td>
<td>4.3</td>
<td>146</td>
<td>14.6</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>B10.D2a</td>
<td>27</td>
<td>12</td>
<td>12</td>
<td>0.44</td>
<td>0.22</td>
<td>0.87</td>
</tr>
<tr>
<td>DBA/2a</td>
<td>48</td>
<td>25</td>
<td>25</td>
<td>0.52</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* ID_{50}: Inhibition of the cytotoxic activity of a monospecific alloantiserum employed at the last point of titration equal to 95% cell death (0 cytotoxic units) by using the eosin micromethod; target cells L1210.
* Cultured L1210 cells.
* L1210 cells grown in ascites of B10.D2 mice.
* Spleen cells.
* Spleen cells of a spontaneous lymphoid cell leukemia of C57BL/6 mice.
* Target cells: spleen cells of C57BL/10 mice.
* Spleens homogenized; cell numbers calculated on the basis of eight spleens equal to 10^9 cells.
* Papain extracts were supplied by Dr. S. Nathenson, through the courtesy of the Transplantation Immunology Branch, NIAID, Bethesda, Maryland.
relatively poor viability (<80%) were utilized for extraction, the recovery of H-2 antigens also was quite low, i.e., less than 3.0 ID₆₀ units/mg protein for H-2.4 and less than 5.0 ID₆₀ units/mg protein for H-2.31.

When our antigen material was compared to a soluble H-2 antigen preparation obtained by papain digestion (kindly supplied by the Transplantation and Immunology Branch, NIAID), the respective specific inhibitory activities per microgram protein were essentially the same. The amount of total antigenic activity extracted per 10⁶ cells with 3 M KCl certainly compared favorably to that obtained by papain digestion (Table II).

Attempts were made to purify further the antigen preparations by gel exclusion chromatography on Sephadex G-100. When from 20 to 30 mg protein of the dialyzed, serologically active KCl extract were subjected to gel exclusion chromatography, a 2-fold increase in inhibitory activity was found for H-2.4 and H-2.31 in those fractions which eluted in the exclusion volume. In addition, a 2-fold increase in inhibitory activity for anti-H-2.31 (H-2Kd) was also observed in fractions eluting at a K_d value of 0.51. The total recovery of protein after Sephadex gel filtration was 80%.

When the pooled, serologically active fractions which eluted in the exclusion volume (from 12 to 18 mg protein) were applied to a DEAE-cellulose column, a further 5-fold increase in inhibitory activity was observed among those fractions which eluted with 0.2 M KCl. The total recovery of protein in the eluate from the DEAE-cellulose column was about 65%. Analysis of these fractions on polyacrylamide gel revealed at best a reduction in detectable protein bands to 6 in comparison to the more than 16 components of the original KCl extract. Upon semi-preparative acrylamide gel electrophoresis, essentially equal amounts of anti H-2d (H-2.21 and H-2.4) inhibitory activity were detected in components with Rf values of 0.36 (H-2.4), 0.46 (H-2.31), and 0.72 (H-2.4). Redistributed of the Rf 0.46 eluates revealed activity in electrophoretic moieties with Rf values of 0.46 and 0.72, respectively.

**Immunogenicity: humoral immune response to H-2d antigens.** For determining if solubilized H-2 antigens maintained their antigenic integrity and biologic function, their immunogenic and immunobiologic efficacy was assessed in the allogeneic system. B10 (H-2KdDd) mice (from 8 to 12), differing at H-2K and H-2D from H-2d antigens, were injected with soluble H-2d antigen (ultracentrifuged 3 M KCl extract from L1210 cells) according to the regimens described in Table I. The sera of individual mice immunized by using regimen A (see Table I) were each tested for the presence of both lymphocytotoxic and precipitating antibodies. For the other immunization regimens, sera from groups of 12 to 15 mice each were pooled. Whenever sera did not show detectable cytotoxic activity in the direct lymphocytotoxic test, they were again tested in a two stage lymphocytotoxic test to exclude possible anticomplementarity. In excess of 200 different sera were examined for H-2d specificities during the course of this study.

Soluble H-2d antigens administered i.v. with two subsequent subcutaneous booster injections did not elicit either cytotoxic or precipitating antibodies. Injection of soluble H-2d antigen (1.0 mg) according to regimen A and B (see Table I) or 0.1 mg antigen according to regimen B led to the formation of cytotoxic antibodies in 10 to 40% of the mice. Immunization according to regimens C and D (see Table I) resulted in the production of cytotoxic antibodies. Whenever complete Freund's adjuvant (CFA) was used, precipitating antibodies were produced in some mice. However, these precipitating antibodies did not correlate with any detectable H-2d specificity.

**Specificity of the humoral immune response.** Table III summarizes the results obtained by direct cytotoxic tests of the sera against lymphocytes from known inbred strains. B10 mice immunized with H-2d antigen may potentially produce antibodies against the following H-2d determinants: H-2.31, 34, 8 and 3 of the K region and H-2.4, 3, 13, 41, 42, 43 and 44 of the D region.

The sera of immunized B10 mice showed reactivity against cells from all mouse strains tested except C3H.JK and B10.S mice. This non-reactivity could imply that the sera of B10 mice contain either none or only very weak antibodies directed against H-2.3 (B10.S) and H-2.44 (C3H.JK). Anti-H-2.8 antibodies were present since the sera did show direct cytotoxic activity against B10.M and B10.A(2R) cells. In
the case of all other positively reacting cells, no single determinant could be held responsible for these reactions since only a gross separation can be made of antibodies directed against H-2K\(a\) or H-2D\(a\) or parts of H-2D\(a\). Thus, the positive reaction against B10.A cells indicates the presence of antibodies against H-2D\(a\) determinants, probably mainly directed against H-2.4 and H-2.13. On the other hand, the positive reaction of B10 anti-H-2\(a\) serum against HTG cells suggests that there are also antibodies present which are directed against H-2K\(d\) determinants (H-2.31 and H-2.8).

Absorption studies. Some of the positively reacting sera described above were absorbed with cells from different mouse strains in an effort to confirm that these antisera contained antibodies directed against single H-2\(d\) determinants of the K or D region. In addition, these absorption experiments were also designed to show whether the antisera produced against soluble H-2\(d\) antigens did also contain antibodies against non-H-2 determinants and/or L1210 tumor-specific antigens. The results shown in Table IV indicate that there were no detectable antibodies against either non-H-2 antigens or L1210 specific antigens. However, antibodies against antigens of both H-2K\(d\) and H-2D\(a\) regions could be detected by absorptions with B10.A (H-2K\(d\)D\(a\)) and HTG (H-2K\(e\)D\(p\)) cells. In addition, antibodies against the H-2D antigen H-2.13 could also be detected. No cytotoxic antibodies were found against H-2.34 (H-2K\(a\)), H-2.3, and H-2.41 antigens.

Precipitating antibodies. Some mice produced antibodies which precipitated soluble H-2\(a\) antigen in the double immunodiffusion test. These precipitating antibodies were only observed in sera of animals which had been immunized with antigen in CFA. The precipitating antibody was not directed against FCS components, since no precipitin line appeared against highly concentrated FCS. Furthermore, these precipitating antibodies showed no clear correlation with H-2 specificities.

**Immunogenicity: cellular immune response to H-2\(d\) antigens.** B10 mice previously sensitized with soluble H-2\(d\) antigen did reject B10.B2 skin in a fashion similar to a second set reaction (Table V). Soluble antigen (0.1 mg) either was injected once or together with two challenge injections (groups A and B). No sensitization could be detected when only a single dose of 10 \(\mu\)g of soluble antigen was injected. B10 mice sensitized against H-2\(d\) antigens did not reject syngeneic skin.

**DISCUSSION**

The serologic data presented indicate that soluble H-2\(d\) antigen extracted from cultured L1210 cells by 3 M KCl possess specific H-2\(d\) antigenic activity in vitro as well as in vivo. With this extraction method, the recovery of soluble H-2\(d\) antigens from cultured cells was as good as that from spleen cells. Gel exclusion and ion exchange chromatography made it possible to achieve a 5- to 7-fold increase in anti-H-2 inhibitory activity for both private antigens of H-2\(a\). However, these antigen preparations were quite heterogeneous when examined by acryl-
TABLE IV
Results of absorption of B10 anti-H-2\(^a\) sera

<table>
<thead>
<tr>
<th>Sera (Regimen)</th>
<th>Absorbing Cells</th>
<th>Target Cells</th>
<th>AD_{50} (Cells \cdot 10^9)</th>
<th>Detectable Determinants</th>
<th>Absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>46-10 (D)</td>
<td>B10. D2</td>
<td>B10. D2</td>
<td>5.0</td>
<td>non-H-2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DBA/2(^a)</td>
<td>B10. A</td>
<td>2.5</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B10. D2</td>
<td>&gt;20.0</td>
<td>H-2K(^a) (^b)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HTG(^a)</td>
<td>&gt;20.0</td>
<td>H-2K(^a) (^b)</td>
<td>+</td>
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<td></td>
<td></td>
<td>DBA/1(^a)</td>
<td>3.0</td>
<td>H-2.34</td>
<td>-</td>
</tr>
<tr>
<td>HTG</td>
<td></td>
<td>HTG</td>
<td>0.6</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B10. D2(^a)</td>
<td>&gt;12.0</td>
<td>H-2D(^a) (^c)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B10. A(^a)</td>
<td>&gt;12.0</td>
<td>H-2D(^a) (^c)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B10. P(^a)</td>
<td>1.2</td>
<td>H-2.41</td>
<td>+</td>
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<td></td>
<td></td>
<td>DBA/1(^a)</td>
<td>2.4</td>
<td>H-2.33</td>
<td>+</td>
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<tr>
<td>46-1 (D)</td>
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<td>5.0</td>
<td>Lsa(^d)</td>
<td>-</td>
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<tr>
<td></td>
<td></td>
<td>L1210</td>
<td>5.0</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Target cells differ at their non-H-2 antigens from the absorbing cells.

\(^b\) H-2K\(^a\), H-2.31, 34.

\(^c\) H-2D\(^a\), H-2.4, 13, 41, 42, 43, 44.

\(^d\) Lsa, L1210 specific antigen.

TABLE V
Skin graft survival time on B10 recipients sensitized with soluble H-2\(^a\) antigen

<table>
<thead>
<tr>
<th>Group</th>
<th>Recipient (No. of Animals)</th>
<th>Initial Dose of Antigen</th>
<th>Challenge</th>
<th>Donor</th>
<th>MST*</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (^a)</td>
<td>B10 (9)</td>
<td>100(^e)</td>
<td>100(^e)</td>
<td>B10. D2</td>
<td>7.3</td>
<td>1.3</td>
</tr>
<tr>
<td>B</td>
<td>B10 (8)</td>
<td>100(^e)</td>
<td>100(^e)</td>
<td>B10. D2</td>
<td>8.5</td>
<td>1.4</td>
</tr>
<tr>
<td>C</td>
<td>B10 (8)</td>
<td>10(^e)</td>
<td>10(^e)</td>
<td>B10. D2</td>
<td>12.0</td>
<td>1.4</td>
</tr>
<tr>
<td>D</td>
<td>B10 (9)</td>
<td></td>
<td></td>
<td>B10. D2</td>
<td>11.0</td>
<td>1.6</td>
</tr>
<tr>
<td>E</td>
<td>B10 (6)</td>
<td>B10. D2 skin</td>
<td></td>
<td>B10. D2</td>
<td>6.7</td>
<td>0.8</td>
</tr>
<tr>
<td>F (^a)</td>
<td>B10 (6)</td>
<td>100(^e)</td>
<td>100(^e)</td>
<td>B10</td>
<td>&gt;60 days (^f)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Mean survival time.

\(^b\) Immunization according to Schedule A.

\(^c\) Injected intravenously.

\(^d\) Injected subcutaneously.

\(^e\) Probability < 0.05.

\(^f\) The fate of these grafts was not followed further.

amide gel electrophoresis since they contained at least six distinct components. Although H-2.31 activity was found in two separate fractions (Kd 1.0 and 0.51) upon gel exclusion chromatography, it is not possible to conclude from these data that separate molecular entities of antigens were involved. In fact, it is more likely that aggregation-disaggregation phenomena account for this observation as well as for the finding of antigentic activities in different electrophoretic moieties. Thus, H-2.4 and H-2.31 activities were found in components with Rf values within a region from 0.36 to 0.46 whereas H-2.4 activity was also detected in a component with Rf 0.72. The hypothesis that aggregation-disaggregation phenomena accounted at least in part for the electrophoretic distribution of H-2.4 and H-2.31 activities was strengthened by the observation that re-electrophoresis of antigenic components with Rf 0.46
resulted in a redistribution of antigenic activity into moieties with Rf values of 0.46 and 0.72, respectively. As far as the relative molecular size of components obtained by DEAE-cellulose chromatography possessing H-2.4 and H-2.3I antigenic activity is concerned, preliminary analyses of radiolabeled immunoprecipitates on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis indicate values of 46,000 and 42,000, respectively. The component (H-2.4) with a relative mobility of 0.72, both in polyacrylamide gels (7.5%) with and without SDS, had a relative molecular size of 35,000.

Our data partially agree with those of Shimada and Nathenson (28) who found H-2a antigenic activity after polyacrylamide gel electrophoresis of papain-solubilized H-2 antigens in components with relative mobilities between 0.36 and 0.4. Our results from gel filtration and DEAE-cellulose chromatography do agree with theirs.

Although we could show that partially purified soluble H-2a antigens exhibited an increased antigenic activity as measured by their capacity to inhibit the cytotoxic activity of specific alloantisera, we could not determine their immunogenicity since we lacked sufficient amounts to carry out the extensive studies made with crude, soluble KCl extracts. All H-2a determinants in the solubilized material remained essentially intact and were not destroyed by the KCl extraction procedure because immunized allogeneic animals produced antibodies against H-2 determinants of antigens from both H-2a regions. The specificity of the antibody response was indicated by direct cytoxicity as well as absorption studies. No antibodies directed either against non-H-2 determinants or L1210 specific antigens (tumor or virus antigens) could be detected.

It would certainly have been most desirable to prepare soluble antigenic extracts from cultured murine lymphoid cells possessing different H-2 specificities and to demonstrate accelerated rejection with proper third party controls. This experiment unfortunately could not be carried out since presently only L1210 (H-2a) cells are available in long-term continuous culture. Nevertheless, it was possible to show a specific cellular immune response with the available antigenic material. Thus, immunization with soluble H-2a antigen led to specific sensitization against H-2a antigens since skin grafts of B10.D2 mice were rejected in a fashion similar to second set reactions. These results concur with previous reports which described the utilization of soluble H-2 antigens prepared by enzymatic digestion (2, 4, 8, 9). Furthermore, soluble H-2 antigens from cultured L1210 cells and from several different mouse spleen cells could be obtained by 3 M KCl extraction in yields that compared favorably with those from papain extracts of murine spleen cells obtained from the Transplantation Immunology Branch, NIH. In this regard, generally higher yields of specific antigenic activity have been reported in 3 M KCl-solubilized extracts than in papain-solubilized antigen preparations also obtained from the Transplantation Immunology Branch (29). The activity of the KCl-solubilized materials was typically that of histocompatibility antigens because it inhibited specifically H-2 alloantisera in vitro and elicited specific humoral as well as cellular immune responses in vivo.

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REFERENCES