Immunosuppressive Agents in Intracellular Infection: Besnoitiosis in Hamsters

H. R. WILSON and J. K. FRENKEL
Department of Pathology and Oncology, University of Kansas School of Medicine, Kansas City, Kansas 66103

Received for publication 10 February 1971

When tested for their activity in suppressing the acquisition of immunity during acute Besnoitia infection of hamsters, antilymphocyte serum (ALS), aminopterin, cyclophosphamide, cortisol, and whole-body irradiation were the most active agents and effectively blocked the development of immunity during 4 to 12 days of immunization. Actinomycin D and chlorambucil were moderately active, and nitrogen mustard, 6-mercaptopurine, and vinblastine exhibited slight immunosuppressive activity. Established immunity was especially labile to cortisol and cortisone administration with generalized Besnoitia relapse occurring consistently in all hamsters; this occurred infrequently during cyclophosphamide treatment. Focal relapse was seen in chronically irradiated and ALS-treated hamsters, but the location of the lesions differed. Irradiated hamsters had lesions in the lungs and brain, whereas ALS-treated hamsters showed splenic relapse. Acquisition of immunity was more sensitive to suppression than established immunity and did not necessarily parallel antibody development and vice versa, emphasizing the importance of cellular over humoral factors in immunity to this intracellular parasite.

Besnoitia jellisoni is an obligate intracellular protozoan parasite resembling Toxoplasma both in its morphology and pathogenic effects in the hamster (2). Utilizing therapeutic control of infection, specific immunity to Besnoitia is developed which can be transferred by cells from immune donors (6).

Corticoid administration delays the immunization process, curtails the ability of cells from immune donors to transfer immunity, and suppresses established immunity to a degree that generalized, fatal Besnoitia relapse occurs consistently (4, 9). The effects of irradiation on acquisition and transfer of immunity are similar to those of corticoids; however, established immunity is quite resistant to radiation (J. K. Frenkel and H. R. Wilson, J. Infec. Dis., in press). Such difference in action suggested that studies utilizing other potentially immunosuppressive agents might contribute to the characterization of the cells involved in this specific cellular immunity.

MATERIALS AND METHODS

Hosts. Female LVG/LAK golden hamsters, 6 weeks of age or older, obtained from Lakeview Hamster Colony, Newfield, N. J., were used in all experiments.

Infection. Hamsters were inoculated subcutaneously between the scapulae with approximately 10⁶ B. jellisoni obtained from pooled mouse ascitic fluid as described previously (6).

Immunization experiments. Prophylactic treatment with sulfadiazine inhibits multiplication of Besnoitia, and the degree of immunity acquired increases with duration of therapy during the first 2 weeks of infection (9). Thus, various degrees of immunity were developed in the two immunization experiments by giving hamsters 90 mg of sodium sulfadiazine per 100 ml in their drinking water for 3, 7, or 11 days beginning the day after infection.

Treatment with potentially immunosuppressive agents was initiated 24 hr before infection and continued through day 20 except as indicated. Cortisol as the free alcohol was used as a known immunosuppressive agent (9).

Chronic infection with immunity. Chronic Besnoitia infection was produced by infecting hamsters and treating them with sodium sulfadiazine as above from day 1 to day 14; they were then challenged subcutaneously with live Besnoitia on day 28. Their immune status was verified by the capacity to survive a subcutaneous challenge of 10⁶ organisms, usually without developing a lesion at the injection site. Since immunity to Besnoitia is of the premunition type (4, 9), hamsters surviving the challenge infection were considered to be both immune and chronically infected.

Potentially immunosuppressive agents were administered, utilizing relapse as an indicator of their potency in comparison with cortisol alcohol.
Autopsy procedure and findings. Complete autopsies were performed on hamsters which died in these experiments to determine the probable causes of death, in particular to differentiate between Besnoitia-related deaths and those due to direct toxic effect of some of the drugs used. Data were obtained from gross findings, lung impression smears, and histological study of all organs. Impression smears were stained with Giemsa. Organs were fixed in Zenker-Formalin solution (Helly’s fluid), and sections were stained with Giemsa. Bacterial cultures were made on blood-agar plates when gross findings at death were not typical of besnoitiosis.

The lesions resulting from acute Besnoitia infection in subcutaneously inoculated hamsters include: pneumonia, meningoencephalitis, and variable degrees of infection-necrosis in the adrenals, liver, spleen, lymph nodes, thymus, bone marrow, or fat.

Typical lesions of relapse included pneumonia and encephalitis, occasionally with lesions in several organs, all a result of the reactivated Besnoitia infection.

Immunosuppressive agents. All cytotoxic drugs were given at near-lethal levels as determined in preliminary chronic administration tests: the dose of a drug was doubled biweekly; the last dose before that which caused drug-related death was used.

(i) Actinomycin D (Cosmegen; Merck Sharp and Dohme) was reconstituted and diluted with sterile distilled water to 0.020 mg/ml; samples were kept frozen at -20 C; 6.0 μg was injected intraperitoneally six times per week.

(ii) Aminopterin sodium (Lederle) tablets were crushed and suspended in 10% gum acacia before use; 100 μg was given by stomach tube six times per week.

(iii) Antilymphocyte serum was produced in a rabbit by injection of LVG/LAK hamster thoracic duct lymphocytes. Initially, 5 × 10⁷ lymphocytes were injected in complete Freund’s adjuvant in all footpads; a month later three daily subcutaneous injections of approximately 10⁷ lymphocytes were given. Weekly, thereafter, the rabbit was alternately bled for serum or given subcutaneous booster injections of thoracic duct lymphocytes. Serum collected from four bleedings was pooled, inactivated, adsorbed with

Table 1. Influence of immunosuppressive agents on the acquisition of immunity to Besnoitia

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Route†</th>
<th>Day of death of individual hamsters after Besnoitia infection‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4⁷</td>
</tr>
<tr>
<td>Expt 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (normal rabbit serum)</td>
<td>0.2 ml, 3X/wk</td>
<td>sc</td>
<td>13 19 23</td>
</tr>
<tr>
<td>Cortisol alcohol</td>
<td>2.5 mg, 2X/wk</td>
<td>sc</td>
<td>11 11 11</td>
</tr>
<tr>
<td>Antilymphocyte serum</td>
<td>0.2 ml, 3X/wk</td>
<td>sc</td>
<td>10 12 13</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>0.1 mg, 3X/wk</td>
<td>ip</td>
<td>12 13 14</td>
</tr>
<tr>
<td>Nitrogen mustard</td>
<td>600 rads, day</td>
<td>sc</td>
<td>10 11 13</td>
</tr>
<tr>
<td>Whole-body irradiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2.5 mg, 2X/wk</td>
<td>sc</td>
<td>27 31 S S</td>
</tr>
<tr>
<td>Cortisol alcohol</td>
<td>0.1 mg, 6X/wk</td>
<td>ip</td>
<td>10 13 15</td>
</tr>
<tr>
<td>Antimoticyn D</td>
<td>2.0 mg, 6X/wk</td>
<td>po</td>
<td>7 11 11</td>
</tr>
<tr>
<td>Aminopterin sodium</td>
<td>6.0 mg, 6X/wk</td>
<td>po</td>
<td>14 16 19</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>6.0 mg, 6X/wk</td>
<td>ip</td>
<td>14 15 16</td>
</tr>
<tr>
<td>Vinblastine sulfate</td>
<td>15 μg, 6X/wk</td>
<td>ip</td>
<td>17 20 21</td>
</tr>
</tbody>
</table>

a ip, intraperitoneal; po, oral (stomach tube); sc, subcutaneous.
b Day of death in boldface indicates non-Besnoitia related death; S, indefinite survival.
c Day sulfadiazine therapy discontinued.
d Mean time to death of hamsters dying of Besnoitia infection.
e Reduced from 30 μg 6X/week on day 7.
normal hamster erythrocytes, and frozen at −20°C until used.

(iv) Chlorambucil (Leukeran; Burroughs Wellcome & Co.) tablets were crushed and suspended in 10% gum acacia before use; 3.0 mg was given by stomach tube six times per week.

(v) Cyclophosphamide (Cytoxan; Mead Johnson Laboratories) was dissolved immediately before use and diluted with sterile distilled water to either 10 or 15 mg/ml. A 10-mg amount given three times per week or 15 mg given two times per week was injected subcutaneously.

(vi) Cortisone acetate and cortisol alcohol (Merck Sharp and Dohme or The Upjohn Co.) were injected subcutaneously as aqueous suspensions; doses ranged from 1 mg given once a week to 2.5 mg given two times a day.

(vii) 6-Mercaptopurine (Purinethol; Burroughs Wellcome & Co.) tablets were crushed and suspended in 10% gum acacia before use. A 6-mg amount given six times a week was administered by stomach tube.

(viii) Nitrogen mustard (Mustargen; Merck Sharp and Dohme) was dissolved and diluted immediately before use with sterile distilled water to 1.0 mg/ml; 0.1 mg was injected intraperitoneally three times per week.

(ix) Vinblastine sulfate (Velban; Eli Lilly & Co.) was reconstituted and diluted with sterile normal saline to 0.1 mg/ml; samples were frozen until use. A 30-µg amount was injected intraperitoneally for six daily doses; then, in view of apparent toxicity, the dose was reduced to 15 µg given six times per week.

(x) Whole-body irradiation was delivered as cobalt-60 gamma irradiation at a target distance of 100 cm, 85 rads/min, for the total dose indicated with each experiment.

**Antibody titration.** Besnoitia antibody titers were determined by using the Sabin-Feldman dye test (29) for Toxoplasma antibody, essentially as outlined by Frenkel and Jacobs (8). In these experiments, Besnoitia organisms were used instead of Toxoplasma, and citrated plasma was used as accessory factor according to the procedure of Wallace (32).

**RESULTS**

**Acquisition of immunity.** Sufficient immunity developed in the nonimmunosuppressed hamsters during 8 days of sulfadiazine control of infection to permit indefinite survival (Table 1, Fig. 1). However, most immunosuppressive agents impaired the acquisition of immunity so severely that many hamsters eventually died of Besnoitia infection, even after 12 days of sulfadiazine control of infection. Thus, hamsters treated with irradiation, with aminopterin, and, in experiment 1, with cortisol alcohol died of acute Besnoitia infection 7 to 8 days after cessation of sulfadiazine therapy. In contrast, the time to death after discontinuing sulfadiazine therapy was considerably delayed in the actinomycin- and especially the chlorambucil-treated hamsters, demonstrating that, although some immunity was acquired in the case of the latter two drugs, it was insufficient to prevent the eventual death of the hamster. Antilymphocyte serum, which

---

**Fig. 1.** Immunosuppressive agents against besnoitiosis of hamsters: suppression of acquisition of immunity. Abbreviations: ACT, actinomycin D; ALS, antilymphocyte serum; AMN, aminopterin sodium; CHB, chlorambucil; CY, cyclophosphamide; Falc, cortisol alcohol; HN₂, nitrogen mustard; 6-MP, 6-mercaptopurine; VLB, vinblastine; WBI, whole-body irradiation.
was initially as active as irradiation or cortisol alcohol, did not inhibit the development of some immunity in the day 12 subgroup, as was the case with cortisol in experiment 2.

Cyclophosphamide showed immunosuppressive activity throughout 12 days of immunization, the effects being partly obscured by general toxic manifestations which were more severe than anticipated. Vinblastine, nitrogen mustard, and 6-mercaptopurine only inhibited the early acquisition of immunity; i.e., treated hamsters in the day 4 subgroup died of Besnoitia infection sooner than the untreated controls.

Toxicity was particularly evident in hamsters treated with cyclophosphamide and vinblastine; the majority of these hamsters appeared to have died of causes unrelated to Besnoitia infection (Table 1). Vinblastine-treated hamsters developed intestinal disturbances (e.g., diarrhea, volvulus, hemorrhage), whereas hamsters treated with cyclophosphamide died with severe bone marrow depression and bronchial obstruction with mucus and desquamated epithelial cells as the principal findings.

**Antibody formation.** The effect of the various agents on the establishment of protective immunity in the hamsters did not necessarily correspond to their effect on anti-Besnoitia antibody development (Table 2). For example, whole-body irradiation and cortisol alcohol depressed antibody development only slightly, yet the hamsters were prevented from effecting a protective immune response; conversely, 6-mercaptopurine depressed the antibody response markedly while immunity became manifest. Actinomycin, chlorambucil, and especially aminopterin and cyclophosphamide depressed both antibody and immunity to Besnoitia; vinblastine did not affect antibody levels or immunity to any degree.

**Relapse of Besnoitia infection.** Of all treatments

---

**Table 2. Influence of immunosuppressive agents on anti-Besnoitia antibody development**

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>Dose</th>
<th>Routeb</th>
<th>Dye test titers on days 19-21c</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol alcohol</td>
<td>2.5 mg, 2X/wk</td>
<td>sc</td>
<td>&gt;1:2,000d</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>6.0 mg, 6X/wk</td>
<td>ip</td>
<td>1:512d</td>
</tr>
<tr>
<td>Aminopterin sodium</td>
<td>0.1 mg, 6X/wk</td>
<td>po</td>
<td>1:16</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>3.0 mg, 6X/wk</td>
<td>po</td>
<td>1:64</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>10 mg, 3X/wk</td>
<td>sc</td>
<td>1:2</td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>6.0 mg, 6X/wk</td>
<td>po</td>
<td>1:8</td>
</tr>
<tr>
<td>Vinblastine sulfate</td>
<td>15 mg, 6X/wk</td>
<td>ip</td>
<td>1:1,024</td>
</tr>
<tr>
<td>Whole-body irradiation</td>
<td>600 rads, day -1</td>
<td></td>
<td>1:512</td>
</tr>
</tbody>
</table>

---

**Table 3. Capacity of various agents to cause relapse of Besnoitia infection**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Routea</th>
<th>Mean time to death (days)b</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antilymphocyte serum</td>
<td>1.0 ml, 3X/wk</td>
<td>ip</td>
<td>14.5</td>
<td>Focal relapse in spleen</td>
</tr>
<tr>
<td>Cortisol alcohol</td>
<td>1.0 mg, 1X/wk</td>
<td>sc</td>
<td>41.8</td>
<td>General relapse, all animals</td>
</tr>
<tr>
<td>Cortisol alcohol</td>
<td>2.5 mg, 2X/wk</td>
<td>sc</td>
<td>19.7c</td>
<td>General relapse, all animals</td>
</tr>
<tr>
<td>Cortisol alcohol</td>
<td>2.5 mg, 2X/day</td>
<td>sc</td>
<td>8.8</td>
<td>General relapse, all animals</td>
</tr>
<tr>
<td>Cortisone acetate</td>
<td>2.5 mg, 1X/wk</td>
<td>sc</td>
<td>40.0d</td>
<td>General relapse, all animals</td>
</tr>
<tr>
<td>Cortisone acetate</td>
<td>2.5 mg, 2X/wk</td>
<td>sc</td>
<td>20.2d</td>
<td>General relapse, all animals</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>10.0 mg, 3X/wk</td>
<td>sc</td>
<td>29.2</td>
<td>General relapse, 2 of 6 animals</td>
</tr>
<tr>
<td>Whole-body irradiation</td>
<td>900 r/</td>
<td></td>
<td>21+</td>
<td>Focal relapses in brain and lung</td>
</tr>
</tbody>
</table>

---

*a Titers not determined for antilymphocyte serum- and nitrogen mustard-treated hamsters.
*b Abbreviations: sc, subcutaneous; ip, intraperitoneal; po, oral (stomach tube).
*c Pooled sera from at least three hamsters.
*d Results from two experiments.
examined, only the corticoids regularly produced fatal Besnoitia relapse (Table 3). A dose of 2.5 mg of cortisol alcohol two times per week was apparently as effective as the equivalent dose of cortisone acetate as judged by the mean time to death of the treated hamsters in each case. Although fatal generalized Besnoitia relapse was seen in two cyclophosphamide-treated hamsters, four of six animals died of causes not related to Besnoitia infection. Chronically irradiated hamsters had only focal Besnoitia relapse in the brain or lungs, an event frequently seen after an accumulation of greater than 900 r; the majority of these hamsters died of bacterial infection.

Hamsters treated with antilymphocyte serum developed a septic peritonitis and probably died from its sequelles. However, at death, areas of splenic necrosis with multiplying Besnoitia located peripherally were observed in all animals; no other significant lesions attributable to Besnoitia were found in these hamsters.

None of the other immunosuppressive treatments employed in immunization experiments produced relapse of Besnoitia infection, even when the treatments were administered at levels which eventually caused death of the hamsters.

DISCUSSION

Survival of a Besnoitia-infected hamster after withdrawal of sulfadiazine therapy is a measure of immunity to this infection (2, 4, 9). With this criterion, all agents examined inhibited the acquisition of immunity during primary Besnoitia infection to some degree. Aminopterin, cortisol, and irradiation were the most active, and most hamsters treated with these agents died of acute besnoitiosis soon after sulfadiazine was discontinued. Antilymphocyte serum was also as active as the above agents in the initial stages of infection; the reasons for the decrease in activity after 12 days of immunization are not clear. Although a foreign protein and presumably antigenic, formation of antibodies to antilymphocyte serum in the recipient animal does not necessarily mean diminution of antilymphocyte serum activity (24).

Considering established immunity, however, only cortisol or cortisone regularly provoked generalized Besnoitia relapse, as has been found after administration of other corticosteroid preparations (4). Established immunity to latent infections appears particularly labile to exogenous hypercorticism. As in chronic besnoitiosis, relapse as a result of corticoid-induced immunosuppression has been demonstrated in toxoplasmosis (1–3), pneumocystosis (7, 26, 27), several fungal infections (5), tuberculosis (14), cytomegalovirus infection (17), and varicella infection (25).

Although a critical part of each of the above established immunities is quite sensitive to hypercorticism, there may be subtle but important differences present, even in the immunities of two closely related organisms (2). Thus, Besnoitia infection relapses more readily than Toxoplasma infection after identical cortisone treatment (4), whereas the reverse has been observed after repeated irradiation (J. K. Frenkel, unpublished data).

However, caution must be observed in interpreting and establishing the general significance of results obtained concerning the dissection of the immune response. In the case of immunosuppressive agents, where an agent is active in one model but differs in activity or is inactive in another, consideration must be given not only to the models per se, but also to the dose, route, and handling of the agent and the antigen by the animals involved. For example, in intracellular infections, vinblastine completely inhibits the immune response to Listeria monocytogenes in mice (30), but, as was shown in the present studies, it has little effect in the Besnoitia-hamster model, even at toxic doses. Further, irradiation is extremely effective both in abolishing transferrable immunity (J. K. Frenkel and H. R. Wilson, J. Infec. Dis., in press) and preventing the development of immunity in Besnoitia-infected hamsters. In contrast, irradiated, lymphocytic choriomeningitis virus-infected mice show less disease than unirradiated controls (28). In the latter case, tissue injury is related to a hypersensitivity response which irradiation diminishes (23), whereas an intact immune system is necessary for survival in besnoitiosis.

Finally, antilymphocyte serum has been found to be particularly effective in inhibiting the development of a variety of cellular immunities ranging from those involved with transplantation rejection to those involved with infectious diseases (12, 20, 22). With respect to infections, administration of antilymphocyte serum has been found to diminish the cellular immune response to viruses (13, 31), bacteria (10, 11, 21), and a nematode infection (18). The effect of antilymphocyte serum on antibody production is not as well agreed on (15, 19); this effect appears to be influenced even by the strain of animal involved (16), again emphasizing the need for careful analysis of discrepant findings before general interpretation.

The present studies demonstrate that acquisition of immunity to Besnoitia is much more vulnerable to a variety of immunosuppressive agents than established immunity. The cellular nature of anti-Besnoitia immunity in hamsters (6; J. K. Frenkel and H. R. Wilson, J. Infec. Dis., in press).
was reaffirmed from the effects of cortisol and irradiation on immunity, but not antibody production, and the reverse situation in 6-mercaptopurine-treated animals. Detailed studies are in progress to apply these findings to the identification of the cells involved in cellular immunity.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-7810 from the National Institutes of Allergy and Infectious Diseases and by training grant GM-1783 from the National Institute of General Medical Sciences.

LITERATURE CITED