MECHANISMS OF RECOVERY FROM A GENERALIZED VIRAL INFECTION: MOUSEPOX

I. The Effects of Anti-Thymocyte Serum

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The response of a host animal to primary viral infection is complex, and the factors essential for recovery from the infection are difficult to define. Immune responses, both cell-mediated and humoral, interferon production, pyrexia, and local changes in acidity and oxygen tension in infected tissues have all been ascribed possible roles in recovery (1). The importance of any single mechanism probably varies in diseases of different pathogenesis or at particular times during the course of infection, and there will sometimes be interaction and synergism between individual factors.

Since neutralizing antibodies and interferon have well documented modes of antiviral activity, they could logically be assigned rather more importance than cell-mediated immunity, which has no clearly demonstrated antiviral mechanism. However, an increasing body of evidence derived from the study of human infants with primary immunological deficiency diseases points to a very important, perhaps even essential role for a thymus-dependent, cell-mediated immune response in recovery from vaccinia virus infection (2). Experimental evidence concerning this area of host–virus relations is meager, but recently Hirsch et al. (3) showed that heterologous anti-thymocyte serum (ATS)[1] caused significantly increased mortality and morbidity from intravenous vaccinia infection in mice. They postulated that this was due to suppression of cell-mediated immunity; however no direct evidence was produced to show that the cell-mediated immune response to vaccinia virus was suppressed by ATS. This was assumed on the basis of an immunosuppressive potency test using lymphocytic choriomeningitis virus (4).

Previously, Allison and Friedman (5, 6) had produced sound evidence that the regression of primary tumours caused by fibroma virus in rabbits was dependent upon cell-mediated immunity. However, this and other work con-

[1] *Abbreviations used in this paper:* AMS, rabbit anti-mouse macrophage serum; ATS, rabbit anti-mouse thymocyte serum; MEM, minimal essential medium; NRS, normal rabbit serum; PBS, phosphate-buffered saline; PFU, virus plaque-forming units; RBC, red blood cells WBC, white blood cells.

cerning virus-induced tumours (7) may not illustrate mechanisms for the control of cytopathic virus infection.

The present series of investigations was designed to elucidate the relative importance of different mechanisms of recovery from a generalized viral infection. For reasons outlined below, mousepox was chosen as the experimental model.

Ectromelia virus, a member of the vaccinia subgroup of the poxvirus group, is a natural mouse pathogen; it causes a generalized disease, mousepox, which is strikingly similar to human exanthems of viral etiology (8). Virulent and avirulent virus strains, and resistant and susceptible mouse strains are available (9). Cell-mediated (delayed hypersensitivity) and humoral immune responses (10) and interferon (11) are generated during infection, and can be readily quantified. Thus, mousepox provides a flexible experimental model in which mechanisms of recovery from a generalized viral infection may be studied. It has not yet been comprehensively exploited for this specific purpose, although it has been the subject of extensive studies which have been reviewed periodically (12–14).

In this investigation, heterologous anti-thymocyte serum has been used in an attempt to clarify the roles of cellular and humoral immunity and interferon production in the process of recovery from a primary infection. The results suggest an essential role for cell-mediated immunity.

Materials and Methods

Animals.—Inbred C57BL mice and outbred mice of the multicolored Walter and Eliza Hall Institute strain (stock mice) were obtained from breeding colonies in this laboratory and used when young adults.

Virus Strain.—Stocks of virulent Moscow strain ectromelia virus (12) were obtained in the form of 0.95% saline suspensions from supernatants of homogenates of infected mouse livers or spleens. Stocks of avirulent Hampstead egg strain ectromelia virus (12) were obtained from infected chorioallantoic membranes of 14-day old chick embryos. Genetron extraction of crude ectromelia virus stocks was sometimes employed to obtain cleaner virus suspensions (15). Semliki Forest virus stock was an homogenate of infected mouse brain in saline.

Portions of all stocks were stored at −70°C and were always dispersed by an ultrasonic probe (Measuring and Scientific Equipment Ltd., London, England) before use.

Cell Cultures.—Continuous line mouse fibroblasts (L929) were grown at 37°C in Eagle's minimal essential medium (MEM) supplemented with folic acid, tryptose-phosphate broth, dextrose, 10% bovine serum, and antibiotics.

Plaque Assays.—L929 cells were seeded into 60 mm Petri dishes (Falcon Plastics, Los Angeles, Calif.) in 5 ml of a modified medium consisting of MEM with 5% heat-inactivated (56°C for 60 min) bovine serum. Confluent monolayers were infected with virus suspended in MEM with 2% fetal calf serum using 0.2 ml per dish for 60 min at 37°C, and then overlaid with 5 ml modified medium containing 0.01% DEAE dextran and 0.75% Bacto agar (Difco Laboratories Inc., Detroit, Mich.). Infected cultures were incubated at 35°C. In the case of ectromelia virus, an additional 5 ml overlay was added after 3 days, and 4 ml staining overlay (1:10,000 neutral red) after 5 days. Plaques were counted at 6 or 7 days. For Semliki Forest virus, 2 ml staining overlay was added after 2 days and plaques counted at 3 days.

Neutralization Tests.—Mouse sera were assayed for anti-ectromelia activity by a plaque reduction method. Samples of Moscow strain ectromelia virus to give 50–100 plaque forming units (PFU) per L929 monolayer were incubated with serial doubling dilutions of serum in MEM with 2% fetal calf serum for 2 hr at 37°C; each mixture was then assayed for PFU on duplicate L929 cultures. Controls included serial doubling dilutions of a known stock antiserum and normal mouse serum. The latter was needed because of enhancement of plaquing efficiency which occurred at high mouse serum concentrations. The end point was taken as the last dilution resulting in 50% reduction of plaque count below the normal mouse serum control.

Test sera were heat-treated at 56°C for 30 min to inactivate the free virus present in specimens from infected mice. This procedure was also found to eliminate nonspecific neutralization of virus by the small amounts of heparin in some sera. The addition of excess complement or the degree of purity of virus stocks used did not affect end points, but one stock of genetron-purified virus was used throughout.

Since the ectromelia virus stocks had a high ratio of mouse-infectious units to PFU (approximately 50:1) it was desirable to ensure that the neutralization of PFU in vitro reflected the neutralization of mouse-infectious units. Therefore a parallel test was set up in which two samples of the same dilutions of a stock mouse antiserum (and appropriate normal mouse serum controls) were allowed to interact with two different concentrations of virus for 2 hr at 37°C. One virus concentration was chosen for the routine plaque reduction test described above. The other was chosen so that when unneutralized it contained a minimal 100% lethal dose for stock mice (about 10 PFU) when a volume of 0.04 ml was injected subcutaneously into the foot. 10 mice were infected for each serum dilution and deaths recorded. All 10 mice in control groups died. The neutralization end point in vivo was taken as the last dilution of antiserum at which less than 5 out of 10 mice died. This occurred at a 1:512 dilution, whereas the parallel in vitro test gave an end point at 1:1024, thus indicating that plaque reduction in vitro is a meaningful assay of neutralization of viral infectivity for the mouse.

Interferon Assay.—The method used involved plaque reduction of Semliki Forest virus and was essentially similar to that of Subrahmanyan and Mims (11) with the exception that L929 cells were used instead of mouse embryo fibroblasts.

To ensure that plaque reduction activity against Semliki Forest virus reflected similar activity against the virulent Moscow strain of ectromelia virus, two parallel assays were performed with a standard preparation of interferon, one with each virus. The assays gave interferon concentrations of 590 units/ml using Semliki Forest virus and 450 units/ml using ectromelia virus.

Anti-Ectromelia Serum.—C57BL mice were immunized with two injections of Hampstead Egg ectromelia virus 8 wk apart. The first injection (10^6 PFU) was given subcutaneously into the foot and the second (5×10^6 PFU) intraperitoneally. All animals were bled 7 days after the second injection, the sera pooled, heat-inactivated at 56°C for 30 min, and stored in portions at −20°C.

Mouse Cell Suspensions.—Thymuses were removed aseptically, cut into pieces, and pressed through stainless steel sieves into MEM. After dissociation of clumps by pipetting, the suspensions were filtered through cotton-wool columns, washed three times in Puck's A saline, and suspended for injection in Puck's A saline containing 10 international units (IU) heparin/ml. Viability of the final suspension was assessed by trypan blue (0.05%) exclusion. It was usually greater than 90%.

Peritoneal cells were obtained by lavage of the unstimulated peritoneal cavity with 4 ml Ca- and Mg-free phosphate-buffered saline (PBS). Red blood cells (RBC) were separated from blood obtained from the retro-orbital venous plexus with a Pasteur pipette containing a small volume of heparinized saline (10 IU/ml).

Anti-Thymocyte Serum (ATS).—Antisera to C57BL mouse thymocytes were raised by the

method of Levey and Medawar (16). Rabbits were given two intravenous injections of 2×10^8 and 10^9 living thymocytes respectively, 14 days apart, and were bled 7 and 9 days after the second injection. Sera were pooled, heat-inactivated at 56°C for 30 min, and stored at −70°C. This ATS pool was shown to reduce morbidity and mortality in mice infected with lymphocytic choriomeningitis virus, an indication of suppression of the cell-mediated immune response (4).

Anti-Macrophage Serum (AMS).—Peritoneal macrophages were harvested from C57BL mice 6 days after the intraperitoneal injection of 3 ml fluid thioglycollate medium (Difco). This increased the yield 5- to 10-fold over normal numbers. The cells were washed, suspended in MEM with 10% fetal calf serum, and dispensed in 5 ml volumes into 60 mm plastic Petri dishes. After 24 hr at 37°C in a humid 5% CO_2 atmosphere, macrophage monolayers had formed on the plastic; nonadherent cells were washed off with saline and the adherent cells removed with a rubber policeman, washed in Puck's A saline, and suspended for injection in Puck's A saline with 10 IU heparin/ml. This procedure produced an inoculum composed of 97% very large macrophages, with smaller cells and occasional RBC making up the remaining 3%. Viability was 70%. Rabbits were given two intravenous injections of 10^8 macrophages 14 days apart and bled 7 and 8 days after the second injection.

Normal rabbit serum (NRS) was obtained from unimmunized animals. Sera were pooled, heat-inactivated at 56°C for 30 min, and stored at −70°C.

Agglutination Tests.—A tube agglutination method similar to that described by Mackaness and Hill (17) was employed for thymocytes and peritoneal cells. Fractions of washed cell suspensions in Ca- and Mg-free PBS at a concentration of 2×10^7 per milliliter were mixed with equal volumes of serial twofold dilutions of test sera in a final volume of 0.2 ml and let stand at 2°C in an ice bath. The cells were resuspended once by vigorous shaking after 30 min and examined microscopically for agglutination at 60 min.

Hemagglutination titers were determined in standard hemispherical well trays using 0.25 ml volumes of serum dilutions to which were added 0.25 ml of 0.5% v/v washed RBC in PBS. RBC were resuspended after 30 and 60 min at 37°C and then left overnight at 4°C before reading.

Immunofluorescence Tests.—Binding of rabbit gamma globulin to mouse thymocytes and peritoneal macrophages was detected by an indirect method.

Washed thymocytes at a concentration of 2×10^7 per milliliter in PBS were incubated at 37°C for 30 min with dilutions of sera. They were then washed twice with large volumes of PBS and finally suspended in a drop of fetal calf serum and smeared on a glass slide. After air-drying for 60 min the smears were fixed for 10 min with acetone, dried, and stored at −20°C.

Peritoneal cells from normal, unstimulated mice were suspended in MEM with 10% fetal calf serum and placed in small slide-ring culture chambers (18) at less than monolayer density and incubated at 37°C for 24 hr in a humidified air atmosphere with 5% CO_2. Cultures were then washed vigorously over a saline fountain (19) to remove nonadherent cells: this resulted in pure, sparse macrophage cultures on the glass slide floor of the chamber, since other cell types had been phagocytized or washed away. The macrophages were then incubated for 30 min at 37°C in MEM containing various concentrations of the rabbit sera to be tested. After vigorous washing over the saline fountain the cultures were dried (60 min) and acetone-fixed (10 min) in the intact culture chambers. Acetone was then removed and the slide and ring components of the chambers separated, thus leaving the fixed macrophages on a glass slide. These preparations were dried and stored at −20°C.

Thymocytes and macrophages were stained at room temperature for 20 min using fluorescein-conjugated goat anti-(rabbit globulin) globulin (Microbiological Associates, Inc., Bethesda, Md.) with 10% rhodamine bovine albumin as a counterstain. The cells were carefully washed for 20 min with two changes of PBS, dried, and mounted in a mixture of 90% glycerol and 10% PBS. They were examined with a fluorescence microscope with camera at-

tachment and photographed using exposure times of 30 to 60 sec with high speed ektachrome film (ASA 160).

Hematology.—Mice were bled from the retro-orbital plexus with calibrated Pasteur pipettes and 0.1 ml of blood from each mouse was diluted with 0.02 ml saline containing 10 IU heparin/ml. Further dilutions were performed with particle-free saline and total RBC were counted in a Coulter Counter (Model B, Coulter Electronics, Hialeah, Fla.).

To obtain total white blood cell (WBC) counts, RBC were first lysed with 0.02% white Saponin (British Drug Houses Ltd., Poole, England) in saline. Differential WBC counts were made on 100 WBC in Wright-stained smears.

Delayed Hypersensitivity in the Footpad.—The general method used has been described previously (20). Approximately 10^6 PFU Moscow ectromelia virus contained in infected spleen homogenate were injected into the right hind footpad in a volume of 0.04 ml. After 3, 6, and 24 hr the thickness of each hind foot was measured to 0.05 mm with dial gauge calipers (Schnelltaster, H. C. Kröplin GmbH, Schluchtern, Hessen, Germany). Since the right and left hind feet of the mouse do not differ in size, the difference between them indicates the level of hypersensitivity. Unimmunized controls were always tested simultaneously to determine the amount of nonspecific inflammatory swelling due to the test injection.

Assay of Virus in Liver, Spleen, Foot, Blood Cells, and Plasma.—Livers and spleens were removed aseptically from individual mice and homogenized in saline at 4°C with a motorized teflon pestle. 2 ml fractions of each homogenate were dispersed by ultrasound and centrifuged lightly to remove gross debris. Supernatants were appropriately diluted in MEM with 2% fetal calf serum for plaque assays and then stored for future use at −70°C. Feet were cut into eight pieces prior to homogenization.

Minimally heparinized blood was obtained from the retro-orbital plexus or from the heart, and the cells and plasma were separated. Blood cells were washed twice in saline and suspended in MEM with 2% fetal calf serum (20% v/v) for storage at −70°C. Suspensions were treated with ultrasound before performing plaque assays. The total blood volume of a mouse in milliliters was assumed to be 10% of body weight in grams.

Plasma was also stored at −70°C and was treated with ultrasound at a 1:10 dilution in MEM with 2% fetal calf serum prior to determining its virus content.

RESULTS

Specificity of ATS In Vitro.—The immunosuppressive activity of ATS is thought to result from its effects on thymus-derived lymphocytes (21). However, before employing ATS as an immunosuppressive agent for investigation of mechanisms of recovery from mousepox, it was necessary to determine its specificity, since reactivity with mouse cells other than lymphocytes might significantly influence the outcome of infection.

Agglutination using C57BL thymocytes, peritoneal cells, and RBC with ATS, NRS, and AMS was chosen as a simple in vitro test; cytotoxic activity was also present, but is not reported since it is unrelated to immunosuppressive potency (22).

On morphological criteria, thymocyte preparations (pooled from 3 normal mice) used in these tests were >99% small lymphocytes; peritoneal cells (pooled from 10 normal mice) were 37% large macrophages, 8% small lymphocytes, and 55% intermediate cells, i.e., medium lymphocytes or monocytes.

The results of agglutination tests are shown in Table I. In the case of thymo-

cytes, ATS gave higher titers than AMS; in the case of peritoneal cells, AMS gave higher titers than ATS, thus suggesting some degree of specificity of ATS for thymocytes. However, both ATS and AMS possessed RBC-agglutinating activity greater than that for either of the nucleated cell types. The consequences of this activity in vivo will be described subsequently.

It is important to note that ATS had little reactivity against peritoneal cells, as previously noted by Mackaness and Hill (17). This suggested that very little anti-macrophage antibody was present, particularly since that proportion of the peritoneal cells which were of small and medium lymphocyte morphology could have accounted for the observed agglutination. Therefore, it was neces-

TABLE I

Agglutinin Titers of ATS, NRS, and AMS against Thymocytes, Peritoneal Cells, and Red Blood Cells

Cell type	Serum	Reciprocal of titer
Thymocytes	ATS	512
	NRS	8
	AMS	64
Peritoneal cells	ATS	32
	NRS	8
	AMS	256
Red blood cells	ATS	1024
	NRS	16
	AMS	512

sary to determine if ATS contained antibodies with affinity for purified macrophages.

Various dilutions of AMS, ATS, and NRS were incubated with purified cultures of C57BL mouse macrophages or thymocyte smears, and rabbit gamma globulin bound to the cells was then detected by staining with fluorescein-conjugated goat anti-(rabbit globulin) globulin.

At a 1:8 dilution of the sera, there was no evidence of macrophage-bound rabbit antibody with NRS; there was slight fluorescence with ATS and marked fluorescence with AMS (Fig. 1). With thymocytes, ATS gave the most pronounced fluorescence, AMS gave slight fluorescence, and NRS none.

These results confirmed those obtained by agglutination tests, but in addition revealed that ATS contained some antibody with affinity for macrophages. The effect of ATS on macrophage function in vivo in relation to ectromelia virus will be described in a later section.

Effects of ATS on Blood Cell Counts In Vivo.—Preliminary tests showed that ATS was not toxic for mice; 0.4 ml could be injected intravenously without

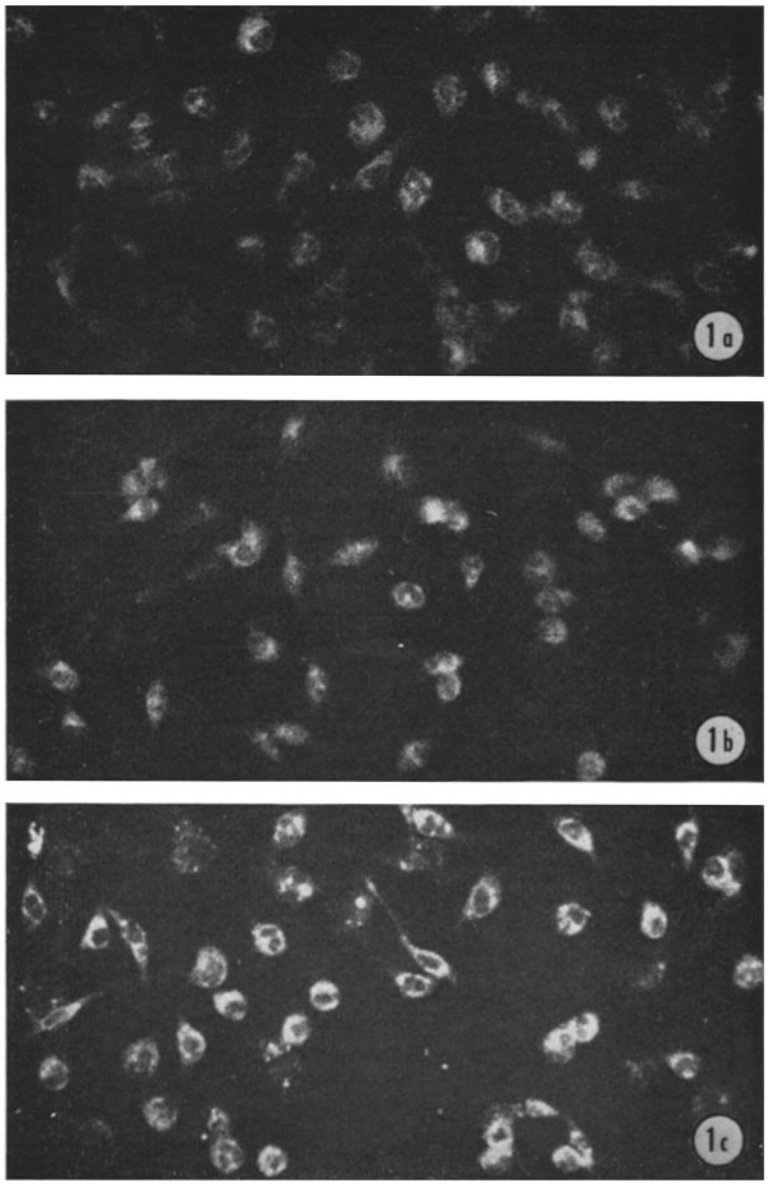

FIG. 1. Mouse peritoneal macrophages stained with fluorescein-conjugated anti-(rabbit globulin) globulin and counterstained with rhodamine bovine albumin following preincubation with 1:8 dilutions of NRS (*a*), ATS (*b*), or AMS (*c*). NRS-treated cells exhibit slight, nonspecific rhodamine fluorescence. Occasional ATS-treated cells show brighter, specific fluorescein emission, while most AMS-treated cells have strong, specific fluorescence. Fluorescence photomicrograph. × 265.

overt ill effects despite its high hemagglutinin titer. Thus it was decided to investigate the effects of ATS on the blood of mice rather than perform empirical absorption with mouse RBC.

Groups of six C57BL female mice 6 wk old were injected with either ATS, NRS, or saline, or were left untreated. The regimen consisted of two subcutaneous injections of 0.2 ml 2 days apart. All mice were bled 3 hr after the second injection and blood cell counts were performed.

The effect of ATS on blood cell counts is shown in Table II. Total WBC counts in ATS-treated mice were less than half those of all control groups. This reduction was found to be highly significant when means were compared using the *t* test. On the other hand, RBC numbers were not significantly affected.

TABLE II

Blood Cell Counts after ATS Treatment*

Treatment‡	RBC	Total WBC	Small lymphocytes	Medium and large lymphocytes	Monocytes	Neutrophil granulocytes
	($\times 10^6$)	($\times 10^3$)	($\times 10^2$)	($\times 10^3$)	($\times 10^2$)	($\times 10^2$)
ATS	7.9 ± 1.9	2.5 ± 0.5§	3.0	1.6	1.5	4.2
NRS	7.5 ± 1.1	6.1 ± 1.0	11.0	4.3	2.4	3.7
Saline	7.4 ± 0.7	7.4 ± 2.2	15.5	5.0	3.0	5.2
Nil	8.6 ± 1.5	6.5 ± 3.4	11.7	4.7	1.3	4.6

* Counts expressed as cells per cubic millimeter of blood. Means of groups of six mice ± standard deviations.

‡ Two subcutaneous injections of 0.2 ml given 51 hr and 3 hr before bleeding.

§ Significantly less than NRS ($0.01 > P > 0.001$), saline ($0.01 > P > 0.001$), and nil ($0.05 > P > 0.02$).

Differential WBC counts allowed arithmetical calculation of an estimate of the numbers of each morphological class of WBC (the four right hand columns of Table II). This revealed that smaller numbers of circulating lymphocytes accounted for the total WBC depletion. Further examination of the blood smears showed that there was no evidence of increased numbers of circulating normoblasts and reticulocytes in ATS-treated mice. Thus, the possibility that large numbers of RBC had been removed from the circulation and replaced by immature forms rapidly generated in hemopoietic tissue seemed unlikely.

Effects of ATS on Organ Weight and Histopathology.—Organs known to be potentially important during infection were removed from groups of six mice 48 hr after a single subcutaneous dose of 0.2 ml ATS, or 3 and 48 hr after the second of two doses given 48 hr apart. Control mice were similarly treated with NRS or saline, or left untreated.

Livers, spleens, and thymuses were weighed and then fixed with 10% formalin in saline together with popliteal lymph nodes.

Examination of hematoxylin- and eosin-stained sections revealed that ATS treatment caused a depletion of lymphocytes from the paracortical area of the popliteal lymph node and from the periarteriolar region of splenic lymphoid follicles. These effects were discernible after a single dose of ATS but were more pronounced after two doses. Liver and thymus were not affected histologically or in weight by ATS treatment. However, there was an increase in spleen weight 48 hr after the second dose of ATS (69 ± 23 mg), NRS (60 ± 15 mg), or saline (59 ± 12 mg) which was significant ($P < 0.05$) when compared with untreated controls (40 ± 9 mg).

The foregoing observations on the effects of ATS on the blood cells and organs of mice are essentially similar to the more comprehensive studies of Taub and Lance (23), with the exception that the present experiments showed no depressive effect of ATS on numbers of circulating RBC or neutrophil granulocytes.

Effects of ATS on Macrophage Function in Vivo.—Since ATS contained slight but detectable anti-macrophage activity (Fig. 1) and since its anti-RBC activity may have promoted phagocytosis of RBC by fixed macrophages of the livers and spleens of ATS-treated mice, functional changes in these littoral phagocytes seemed possible (24). During natural mousepox, infection of the liver and spleen follows uptake of virus from the blood by these macrophages (12). Thus, events in vital target organs are critically dependent upon macrophage function, and for this reason it was important to determine the fate of circulating ectromelia virus in ATS-treated mice.

Groups of 20 C57BL male mice 6 wk old were treated with either ATS or NRS (two subcutaneous injections of 0.2 ml 2 days apart) and infected intravenously with approximately 2×10^5 PFU Moscow strain ectromelia virus 3 hr after the second ATS or NRS injection. Four mice of each group were bled from the retro-orbital plexus 5 min after intravenous infection, and their livers and spleens were removed and snap frozen in liquid nitrogen within 7 min of infection. Livers and spleens of further groups of four mice were taken after 4 hr, 10 hr, 24 hr, and 48 hr. PFU in individual bloods and organs were determined.

The results are given in Table III. Over 99% of virus had been cleared from the blood of both ATS- and NRS-treated mice 5 min after intravenous injection. By 7 min, less than 10% of injected virus could be located in the livers and spleens. Since it has been demonstrated by Mims (25) that the mouse liver takes up over 90% of Moscow ectromelia virus within 5 min of intravenous injection, at which time the virus is located within Kuppfer cells, it is possible that the failure to detect more virus in this experiment was due to a rapid uncoating of virus within littoral macrophages. By 4 hr after injection, PFU numbers were beneath the technical limits of detection in both liver and spleen (at less than 0.1% of the injected dose). By 10 hr, virus was again detectable.

At this time the fluorescence studies of Mims (26) showed that transmission of virus from infected liver macrophages to liver parenchyma cells could be first seen. Rapid multiplication of virus in both liver and spleen then occurred over the next 38 hr. Through all phases of infection, blood clearance, eclipse, and multiplication, there was no significanct difference (using t tests) between the levels of virus in the organs of ATS- and NRS-treated mice, thus indicating

TABLE III

Distribution and Behavior of Moscow Strain Ectromelia Virus after Intravenous Injection of 2×10^5 PFU*

Time after infection	Treatment‡	Blood	Liver	Spleen
5 min	ATS	2.5 ± 0.5		
	NRS	2.5 ± 0.2		
7 min	ATS	—	4.1 ± 0.2	1.8 ± 0.3
	NRS	—	4.0 ± 0.1	1.5 ± 0.2
4 hr	ATS	—	<2.1	<0.70
	NRS	—	<2.1	<0.70
10 hr	ATS	—	2.9 ± 0.1	2.1 ± 0.2
	NRS	—	2.8 ± 0.3	2.2 ± 0.2
24 hr	ATS	—	5.9 ± 0.3	4.8 ± 0.2
	NRS	—	6.3 ± 0.3	5.2 ± 0.3
48 hr	ATS	—	8.3 ± 0.4	7.4 ± 0.1
	NRS	—	8.1 ± 0.2	7.3 ± 0.1

* Expressed as mean log PFU per organ ± standard deviations in groups of four mice.

‡ Two subcutaneous injections of 0.2 ml given 51 hr and 3 hr before injection of virus.

that no important functional defect in the macrophages of the liver and spleen was caused by ATS treatment.

Effect of ATS on the Course and Outcome of Primary Mousepox.—Preliminary experiments showed that even a single subcutaneous injection of 0.2 ml ATS caused over 80% of C57BL mice to die from a subcutaneous infection with a dose of Moscow strain ectromelia virus which killed none of the controls treated with NRS or saline, or left untreated. The single injection of ATS could be given 2 days before, on the day of, or 2 days after infection.

Fenner (12) showed that subcutaneous infection in the foot resulted in a disease indistinguishable from natural mousepox, and that the infected foot could be used conveniently to quantitate infection at the portal of virus entry. Thus this route of infection was employed in the present experiment.

A group of 26 C57BL female mice 8 wk old was treated with two doses of 0.2 ml of ATS given subcutaneously 2 days and 3 hr before infection. A control group was similarly treated with NRS. All animals were infected subcutaneously in the right hind foot with 10^3 PFU Moscow strain ectromelia virus. 10 mice of each group were set aside to assess mortality, and 4 mice of each group were sacrificed 4, 6, and 7 days after infection. PFU in the infected foot, liver, spleen, blood cells, and plasma were determined for individual mice as were serum

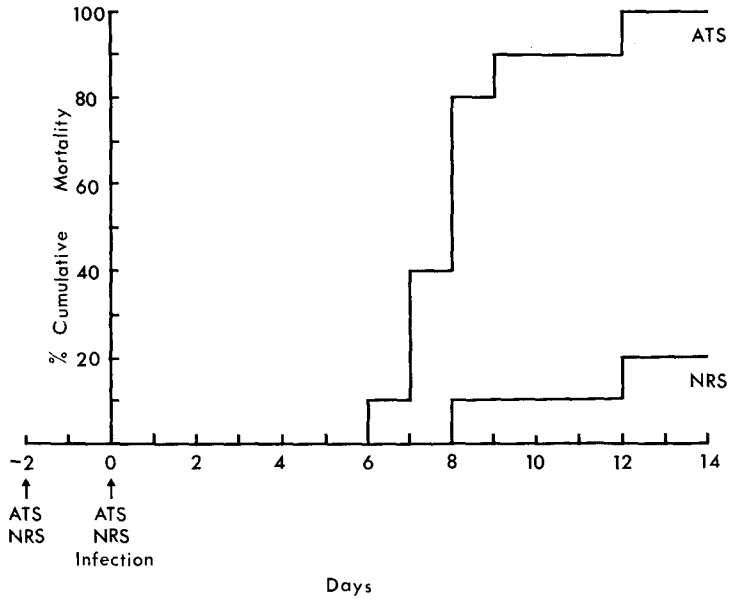

FIG. 2. Cumulative mortality in ATS-treated and NRS-treated mice after subcutaneous infection with 10^3 PFU virulent, Moscow strain ectromelia virus. Two injections of 0.2 ml of ATS (or NRS) were given 51 hr and 3 hr before infection.

neutralizing antibody levels. Spleen homogenates of mice sacrificed on days 4 and 6 were pooled and assayed for interferon. The spleen was chosen for this purpose because it is a major source of interferon during mousepox (11) and provides a relatively "clean" homogenate for processing.

Cumulative mortality is shown in Fig. 2 where it is apparent that ATS treatment caused a highly significant increase in mortality ($\chi^2 = 10.2$, $0.01 > P > 0.001$). The probable explanation for this is revealed in Table IV which shows that the liver (a target organ vital for life), spleen, and blood of ATS-treated mice consistently contained more virus than NRS-treated controls on the 6th and 7th days after infection, although foot titers of both were similar. The viremia seemed to be largely cell-associated as reported previously by Mims (14).

The splenic interferon response is shown in Table V. ATS-treated mice had higher levels than controls, possibly due to the larger stimulus of increased virus growth. However, no neutralizing antibody could be detected, even on the 7th day (Table V) and too few ATS-treated mice survived beyond this time to allow meaningful comparison with controls. Furthermore, the primary foot infection precluded the footpad test for delayed hypersensitivity. Thus, it was decided to use avirulent Hampstead egg strain virus to allow ATS-treated mice to survive long enough for parallel studies of their delayed hypersensitivity and neutralizing antibody responses.

TABLE IV

Virus Content of Tissues after Subcutaneous Infection with 10^3 PFU Moscow Strain Ectromelia Virus*

Day after infection	Treatment‡	Liver	Spleen	Foot	Blood	
					Cells	Plasma
4	ATS	4.5 ± 0.3	5.7 ± 0.5	6.5 ± 0.2	2.8 ± 0.1	2.3 ± 0.2
	NRS	4.6 ± 0.7	5.2 ± 1.4	6.7 ± 0.1	2.6 ± 0.4	2.2 ± 0.5
6	ATS	6.7 ± 0.8	7.9 ± 0.2	7.3 ± 0.2	4.8 ± 0.4	4.2 ± 0.5
	NRS	5.6 ± 0.8§	6.3 ± 2.1	7.1 ± 0.2	3.4 ± 1.0§	3.0 ± 1.1
7	ATS	8.5 ± 1.7	8.9 ± 0.4	7.2 ± 0.2	5.6 ± 0.8	5.4 ± 0.9
	NRS	5.0 ± 2.8	5.4 ± 1.7§	7.8 ± 0.3	3.6 ± 1.8	2.5 ± 1.5

* Expressed as mean log PFU ± standard deviations in groups of 4 mice.

‡ Two subcutaneous injections of 0.2 ml given 51 hr and 3 hr before infection.

§ Significantly less than ATS group ($P < 0.05$).

The Effects of ATS on the Delayed Hypersensitivity and Neutralizing Antibody Responses to Ectromelia Virus.—A group of 38 C57BL female mice 8 wk old were treated with two doses of 0.2 ml ATS given subcutaneously over the left side of the thorax 2 days and 3 hr before infection. A similar control group was treated with NRS. 23 mice of each group were then infected subcutaneously over the right side of the thorax with 5×10^5 PFU of Hampstead egg strain ectromelia virus. On days 5, 7, and 9 after infection, separate subgroups of five ATS- and five NRS-treated mice for each day were tested for delayed hypersensitivity.

Five ATS- and five NRS-treated mice which had not been infected were included in this test as controls indicating nonspecific reactivity on each day. Since the delayed hypersensitivity test involved the injection of 10^6 PFU of virulent Moscow strain ectromelia virus into the foot, these mice provided a concurrent test of the effect of ATS on mortality.

On days 8 and 10 after infection, separate subgroups of four ATS- and NRS-treated mice were bled for neutralizing antibody assays. These times were chosen on the basis of the preceding experiment and previous work by Schell (9) who showed that little or no antibody could be detected before the 8th day after infection with Hampstead egg strain ectromelia virus. Livers and spleens for PFU assays were taken from four mice of each group on days 6, 8, and 10 after infection; on day 6, these organs were taken from mice used in delayed hypersensitivity tests, and on days 8 and 10 from mice which had been bled for antibody assays.

The delayed hypersensitivity and neutralizing antibody responses, and the virus content of organs, are shown in Table VI. The delayed hypersensitivity

TABLE V

Interferon and Neutralizing Antibody Responses after Subcutaneous Infection with 10^3 PFU Moscow Strain Ectromelia Virus

Day after infection	Treatment*	Interferon (Units per spleen)‡	Neutralizing antibody§
4	ATS	15	<5
	NRS	5	<5
6	ATS	20	<5
	NRS	5	<5
7	ATS	—	<5
	NRS	—	<5

* Subcutaneous injections of 0.2 ml given 51 hr and 3 hr before infection.

‡ Derived from pools of four spleens.

§ Reciprocal of titer.

response (24 hr) was markedly depressed in ATS-treated mice so that it reached significant levels by day 9, 4 days later than in controls which gave good reactions when tested on day 5. On the other hand, immediate hypersensitivity (3 hr) was not found in either ATS- or NRS-treated mice until day 9 when it was significantly greater in NRS-treated animals. However, virus-neutralizing activity of sera taken on days 8 and 10 was clearly unimpaired by ATS-treatment.

The apparent discrepancy between the neutralizing antibody response and immediate hypersensitivity, which is also presumably antibody-mediated, could be due to the different test materials employed for measuring each response. Neutralization of virus depends upon interaction between antibody and accessible surface antigens of the infectious particle; a relatively pure preparation of virions was found suitable for detecting this interaction and was routinely employed in the assay. However, the material injected for hypersensitiv-

ity tests was a crude homogenate of infected mouse spleens which contained both virions and other virus-specified antigenic products of infected cells, such as internal structural components of the virion or nonstructural proteins like the hemagglutinin and various enzymes. It is possible that the virions comprised a small proportion of the antigenic spectrum of the homogenate, so that they provided insufficient antigenic stimulus for an immediate hypersensitivity reaction. Alternatively, the class of antibody directed at virion surface antigens

TABLE VI

Neutralizing Antibody and Delayed Hypersensitivity Responses after Subcutaneous Infection with 5 × 10^5 PFU Hampstead Egg Strain Ectromelia Virus

Day after infection	Treatment*	PFU‡		Neutralizing§ antibody	Hypersensitivity‖		
		Liver	Spleen		3 hr	6 hr	24 hr
5	ATS	—	—	—	0.2 ± 0.4	0	0
	NRS	—	—	—	0	0.2 ± 0.3	1.8 ± 0.4¶
6	ATS	4.4 ± 0.5	6.0 ± 0.1	—	—	—	—
	NRS	<2.4	1.2 ± 0.5	—	—	—	—
7	ATS	—	—	—	0.1 ± 0.2	0	0.3 ± 0.4
	NRS	—	—	—	0.3 ± 0.7	1.1 ± 0.8¶	2.8 ± 1 2¶
8	ATS	3.2 ± 0.5	5.7 ± 0.6	25 ± 10	—	—	—
	NRS	<2.4	<0.7	17.5 ± 5	—	—	—
9	ATS	—	—	—	1.3 ± 0.9	1.4 ± 1.0	2.2 ± 1.0
	NRS	—	—	—	3.8 ± 1.5¶	4.4 ± 1.5¶	4.9 ± 1.5¶
10	ATS	<2.4	4.0 ± 0.9	17.5 ± 5	—	—	—
	NRS	<2.4	<0.7	22.5 ± 13	—	—	—

* Two subcutaneous injections of 0.2 ml given 51 hr and 3 hr before infection.

‡ Mean log PFU ± standard deviations in groups of four mice.

§ Means of reciprocals of titers ± standard deviations in groups of four mice.

‖ Means of increases in foot thickness (in 0.1 mm units) ± standard deviations in groups of five mice. The 24 hr readings represent delayed hypersensitivity.

¶ Significantly more than ATS groups ($P < 0.05$).

which was detectable in 8 day sera may not be an efficient mediator of immediate hypersensitivity reactions.

The nonspecific inflammatory response to the foot test injection in uninfected mice was similar in both ATS- and NRS-treated groups at all times, so that it seems unlikely that anti-inflammatory qualities of the ATS (27) contributed to the depression of the hypersensitivity reactions. Thus, the reduced reactivity probably resulted from suppression of the immune response to ectromelia antigens.

Virus grew far more in the livers and spleens of ATS-treated mice than in controls (Table VI). The failure to control virus growth was also reflected in mortality data from the mice acting as controls in the delayed hypersensitivity

test. 4 out of 15 NRS- and 14 out of 15 ATS-treated mice died. Thus, ATS again caused a highly significant increase in mortality ($P < 0.001$) even though the last ATS injections preceded infection by intervals of 5–9 days.

DISCUSSION

The ATS used in these experiments cross-reacted with RBC and macrophages in vitro (Table I). However, this activity did not produce detectable side effects in vivo at dosages which dramatically increased susceptibility to mousepox. Mice could tolerate large intravenous injections of ATS; subcutaneous inoculation caused depletion of lymphocytes from thymus-dependent areas (23) of the popliteal lymph node and spleen, and reduced the numbers of circulating lymphocytes without affecting other cell types in the blood (Table II). Most importantly, the clearance of ectromelia virus from the blood, and its subsequent distribution and behavior in the liver and spleen over a 48 hr time period was not influenced by ATS treatment (Table III). Thus, since the innate resistance of these important target organs was not altered, the effects of ATS on the course and outcome of infection could be attributed to depression of acquired mechanisms of recovery.

The early studies of Fenner (12) on the pathogenesis of mousepox using virulent virus in susceptible outbred mice showed that the natural route of infection was through skin abrasions. After local multiplication in the skin and in draining lymph nodes, there was a primary viremia. By 3 days, infection of the liver and spleen was detectable, presumably as a consequence of uptake of blood-borne virus by littoral macrophages, as later shown by Mims (25). Rapid multiplication of virus then occurred in these organs and probably contributed to a secondary viremia which in turn led to extensive focal infection of the skin. If the mouse survived beyond 7 or 8 days after infection, a rash developed at these skin foci and then healed within a few more days. Deaths could be attributed to unchecked growth of virus and massive necrosis in the liver (28). However, in survivors the rate of growth of virus in the spleen began to decrease by the 6th day of infection (10) which suggested the onset of recovery mechanisms by this time. In contrast, virus growth in the foot, the portal of entry in these experiments, did not slow for another 4 days (10).

The current investigation using both virulent and avirulent virus strains and more resistant C57BL mice has confirmed and extended these findings. Since ATS treatment caused a highly significant increase in mortality rate in mice infected subcutaneously with virulent Moscow strain ectromelia virus (Fig. 2), it provided a means of determining the time of onset of recovery mechanisms. For example, ATS-treated mice began to die 6 days after infection, indicating that NRS-treated controls (most of which survived) must have generated effective recovery mechanisms before this time. This is further illustrated by the fact that more virus was found in the livers, spleens, and

blood of ATS-treated mice on the 6th and 7th days after infection than in the organs of controls (Table IV). Since organ levels on the 4th day were similar, it follows that the innate resistance of the mouse was not changed by ATS, and that acquired recovery mechanisms began to operate between 4 and 6 days after infection. Table IV also shows that these mechanisms did not control virus in the foot as effectively as in the spleen and liver, perhaps reflecting relative inaccessability of virus located in foot tissues.

We can now question the nature of the defect in recovery mechanisms caused by ATS. The host responses measured in the present experiments may be evaluated in terms of their presence or absence at the time of onset of recovery, and their susceptibility to impairment by ATS. Table V shows that the splenic interferon response, although present at times relevant to recovery, was certainly not depressed by ATS. Thus, it appears unlikely that interferon plays a major role in recovery.

There was also no impairment of the neutralizing antibody response of ATS-treated mice to avirulent Hampstead egg strain virus (Table VI) although their recovery mechanisms were unequivocally defective as indicated by increased multiplication of avirulent virus in target organs and increased mortality after infection with virulent virus. Furthermore, no neutralizing antibody had appeared in circulation by late on the 7th day after infection with virulent virus; that is at least 2 days later than the onset of recovery in the spleen and liver (Table V). Similarly, immediate hypersensitivity to ectromelia antigens (Table VI), which is also presumably antibody-mediated, was not at significant levels 7 days after infection with avirulent virus, although ATS did cause a significant depression in the levels of reactivity 9 days after infection. Superficially, these results are strong evidence against an important role for antibodies in recovery. However, it is possible that during mousepox infection, antibody is being produced in lymphoid tissues several days before it is detectable in the circulation. The absence of circulating antibodies in the early stages of infection might then be explained by their rapid absorption by excess viral antigens in infected tissues (29, 30). This also implies that excess free virus would remain available to promote further spread of infection, but it is conceivable that antibody could make some contribution to the control of infection before becoming detectable in the blood. The magnitude of this contribution cannot be accurately assessed in the present experiments, although the fact that excessive growth of virus occurred in ATS-treated mice in the face of what appears to be a normal neutralizing antibody response (Table VI) makes it seem improbable that antibody is a key factor in recovery. This question will be examined more closely in a future paper.

In contrast to its failure to affect interferon or neutralizing antibody production, ATS caused a significant delay in the cell-mediated immune response so that ATS-treated mice took 9 days to generate a level of delayed hypersensitiv-

ity to ectromelia virus antigens already present by 5 to 7 days in NRS-treated animals (Table VI). This is in keeping with other evidence which indicates that ATS may selectively depress thymus-dependent immune responses (which include cell-mediated immunities and humoral antibody responses to certain antigens in some animal species) while leaving thymus-independent antibody responses intact (4, 21, 31). Furthermore, cell-mediated immunity was detectable at a time compatible with a role in recovery. The significant delayed hypersensitivity reaction following a test injection on day 5 after infection indicated the availability of significant numbers of specifically reactive lymphocytes at this time. There is now considerable evidence from other experimental systems which shows that the cell-mediated response in mousepox is not uniquely early. The studies of Turk (32) on the cytodynamics of delayed hypersensitivity responses to skin sensitizing agents suggested that specifically reactive small lymphocytes were generated in responding lymph nodes of guinea pigs by the 5th day after antigen administration. Furthermore, Allison has obtained delayed hypersensitivity reactions to rabbitpox (7) and fibroma viruses (5, 6) in rabbits 4 or 5 days after infection, while delayed hypersensitivity to some bacterial antigens and associated acquired resistance mechanisms are well established in mice within 4 days (33).

The foregoing evidence, which suggests the potential importance of a thymus-dependent response in recovery from mousepox, is in accord with the finding of Subrahmanyan (34) that neonatal thymectomy resulted in increased mortality of susceptible outbred mice after infection with avirulent Hampstead egg strain ectromelia virus. However, Subrahmanyan also showed that this effect could be abrogated by feeding the mice antibiotics from birth, and that mortality of thymectomized mice from a low pathogen colony was unaltered. This suggested a complex interaction between the thymus-dependent immune system, bacterial flora, and the virus which remains unexplained.

Thus, the available evidence would support the contention that cell-mediated immunity plays an essential role in recovery from mousepox, and that the interferon and antibody responses are of secondary importance. Similar conclusions have been drawn from studies with other poxivruses (3, 5, 6) as detailed in the introduction of this paper.

Future articles will present evidence derived from a positive approach to the problem of recovery from mousepox employing transfer of immune spleen cells, immune serum, and interferon to preinfected recipients, and also evidence concerning the possible nature of antiviral mechanisms generated by the cell-mediated immune response.

SUMMARY

Agglutination and immunofluorescence tests in vitro showed that the ATS used in these experiments cross-reacted with macrophages and RBC. However,

ATS was not toxic in vivo, and small doses given subcutaneously depleted thymus-dependent areas of lymphoid tissues and selectively depressed blood lymphocyte counts without affecting other cell types in the blood. Furthermore, the function of littoral macrophages as indicated by the clearance of blood-borne virus and its subsequent behavior over a 48 hr period in the liver and spleen was not changed by ATS. Thus, the innate resistance of these vital target organs was not depressed. A similar regimen of subcutaneous ATS caused a highly significant increase in mortality from mousepox with an associated failure to control virus growth in the liver and spleen which was manifest by 6 days after infection. The interferon and neutralizing antibody responses were not impaired in ATS-treated mice, but the cell-mediated immune response was significantly suppressed. This evidence, and consideration of the timing of these host responses during the course of infection in relation to the control of virus growth in the liver and spleen, led to the conclusion that cell-mediated immunity probably contributed an essential acquired recovery mechanism. However, no evidence was obtained concerning the nature of this antiviral mechanism.

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