PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 165, 107–113 (1980)

Coxsackievirus B3-Positive Mononuclear Leukocytes in Peripheral Blood of Swiss and Athymic Mice during Infection (40942)[1]

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Abstract. Coxsackievirus B3 viremia in infected 14-day-old Swiss and young adult Nude mice is predominantly a plasma viremia. Despite high titers of virus in plasma, separated suspensions of red blood cells and polymorphonuclear leukocytes contain no virus. Virus-positive mononuclear leukocytes in blood, on the other hand, may be present from the second to the fourth days of infection. Virus-positive mononuclear leukocytes may persist after virus is no longer in the plasma. Our studies do not definitively identify the mononuclear population in blood which is virus-positive.

Viremia is common early in human enterovirus infection and manifestations of disease involving the skin, central nervous system, heart, and pancreas are dependent upon transit of virus through the blood (1). However, the distribution of enteroviruses among the cellular components of blood during viremia, to our knowledge, has not been studied. An interesting relationship of coxsackievirus B3 with mononuclear leukocytes has been found.

Materials and Methods. Mice. Swiss ICR and, for certain experiments, Nude (Nu/Nu) athymic mice were used. Pregnant Swiss ICR mice were obtained at term from the Charles River Breeding Laboratories (Wilmington, Mass.). After delivery each mother with its litter was kept in a separate cage. Nude athymic mice 4 to 5 weeks old were obtained from an established colony maintained in the Department of Immunology and Microbiology of Wayne State University School of Medicine. This colony originated from a heterozygote of Nude and a nine-generation BALB/c backcrossed population initially obtained from Dr. Donald C. Shreffler (Washington University, St. Louis, Mo.). These athymic mice were the youngest animals available to us.

Nude mice 4 to 5 weeks of age were kept in polycarbonate cages with fitted bonnets. Cages, bedding, feed, and water were autoclaved and under a horizontal laminar air flow, fresh food and water were given. Nutrients lost by autoclaving were not replaced. After inoculations with virus, nude mice were removed from their protected environment and fed ordinary tap water and Rockland rat chow.

Experimental infection. Swiss ICR 14-day-old mice and 4- to 5-week-old Nu-Nu mice were injected intraperitoneally with 0.05 ml of coxsackievirus B3 (Nancy strain) containing 10^{4-6} TCD_{50}/ml which had been prepared in vero cell tissue cultures by standard methods (2). Younger nude mice often do not survive outside of a protected environment. Although we would have preferred to use athymic mice which were 14 days old, weanling mice of this age were not available to us. Control animals were similar to infected ones except that they were inoculated with sterile Eagle's medium (EM). At various times after inoculations, animals in groups of 5 to 10 were anesthetized with ether and exsanguinated by cutting deeply into an axillary vein. Heparinized blood was collected from individual animals for determination of total and differential white blood cell counts, but

[1] Supported by grants from the Michigan Heart Association, the Skillman Foundation, and The Michigan Foundation for Infectious Diseases.

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0037-9727/80/090107-07$01.00/0

in all other studies which require a greater volume, blood was pooled from groups of 5 to 10 animals.

Postmortem examinations in Nu/Nu mice infected with coxsackievirus B3 were done at times of sacrifice. Hearts, pancreases, thymuses (Swiss mice), spleens, and femurs were removed aseptically. Heart and pancreas were processed for virus isolation as described earlier (3). Other portions of the spleen, thymus, or suspensions of cells from bone marrow to be used in cell separations were kept in sterile chilled RPMI 1640 medium (Grand Island Biological Company, Grand Island, N.Y.) containing 10% fetal calf serum.

Interferon and neutralizing antibodies. We assayed plasma interferon in Nu/Nu coxsackievirus B3 infections in mouse embryo fibroblasts using vesicular stomatitis as challenge virus as described previously (3).

Type-specific antibody titers were determined using microtiter wells of vero tissue cultures. With EM twofold dilutions of plasma were prepared. Two-tenths milliliter of diluted plasma was mixed with 0.2 ml of coxsackievirus B3 containing 200 TCD_{50}/0.1 ml. The mixtures were incubated (28°, 18 hr) in stoppered test tubes. Controls similarly incubated included mixtures consisting of (a) 0.2 ml of virus (200 TCD_{50}/0.1 ml) plus 0.2 ml EM, (b) 0.2 ml of EM plus 0.2 ml of normal mouse plasma, and (c) 0.2 ml of virus (200 TCD_{50}/0.1 ml) plus 0.2 ml of normal mouse plasma. Following completion of the incubation period, 0.1 ml of each mixture was inoculated into three microtiter wells. The neutralizing antibody titer was the highest dilution of plasma completely inhibiting coxsackievirus B3 cytopathic effects when virus controls had destroyed the sheeted cells in their respective wells. Microtiter wells from plasma controls remained intact (3).

Total and differential white blood cell counts and isolation of components in peripheral blood. Immediately after bleeding, using Wright's stain, total and differential white blood cell counts from individual mice were done. On each autopsy day, 5 ml of pooled heparinized blood from five similar mice were layered over Hypaque–Ficoll (4). The Hypaque–Ficoll had been passed through a 0.22-μm Millipore filter. Plasma, mononuclear cells (monocytes, lymphocytes) and platelets, and erythrocytes with granulocytes were separated by centrifugation at 400g for 40 min at 4°. Mononuclear and red blood cell layers were sampled with a Pasteur pipet and stained with Wright's stain. The mononuclear layer contained 5×10^3 cells/ml which were approximately 96% lymphocytes and 4% monocytes. Platelets were also present. The red blood cell layer also contained polymorphonuclear leukocytes. Separated mononuclear and red blood cells were washed three times in EM with 2% fetal calf serum. For attempts at virus isolation, 0.1 ml from whole blood, plasma, tissues, or cell suspensions containing 10^5 to 10^6 mononuclear cells per milliliter was diluted logarithmically in Eagle's medium onto four vero cell tissue cultures grown on microtiter plates (Falcon Plastics, Los Angeles, Calif.). Plates were incubated (35°) and examined daily for 1 week for cytopathic effects. Virus titers were calculated by the method of Reed Muench. Cytopathogenic agents isolated from plasma were identified as coxsackievirus B3 by neutralization tests (5). This was done with each new animal experiment.

Virus was recovered in vero tissue cultures from certain mononuclear cell preparations during infection. In an effort to identify the specific mononuclear cell(s) with which coxsackievirus B3 interacted, the following studies were done.

Separation of cellular components in peripheral blood. Infection and autopsies were performed as before, but the Ficoll concentration was increased to 14% to enhance the purity of the mononuclear layer (6). Plasma, mononuclear cells, and erythrocytes with polymorphonuclear leukocytes were separated by centrifugation at 400g for 40 min at 20°. With a Pasteur pipet a small sample from the mononuclear and erythrocyte layers was taken for Wright's staining. The erythocyte layer was washed three times with RPMI 1640 medium and kept along with plasma at 4° for quantitative virus culture.

Mononuclear cells were washed twice and resuspended in 2 ml of sterile RPMI

1640 containing 10% fetal calf serum. This suspension was layered onto a 15 × 60-mm sterile plastic petri dish (Falcon Plastics, Los Angeles, Calif.) and incubated at 37° in 5% CO_2 for 120 min. Supernatant and nonadherent cells were decanted into a sterile centrifuge tube. This process was repeated with fresh medium. Petri dishes were washed once more and the remaining adherent cells were lifted from the plates by gentle scraping with a rubber policeman. Adherent cells were then suspended in RPMI 1640 (7). Wright stains of both the adherent and nonadherent cells showed that each fraction contained >95% lymphocytes. The adherent lymphocytes at the Wright stain were consistent with "stimulated" B cells which are known to adhere to petri dishes along with monocytes (6, 7).

Separation of leukocytes from thymus, spleen, and bone marrow of Swiss mice. With aseptic technique, pooled thymuses were placed on a sterile stainless-steel mesh over a petri dish containing RPMI 1640–10% fetal calf serum. Thymocytes were freed by gently teasing the organs through the mesh. Cells were washed three times and suspended in RPMI 1640. Using a hemocytometer, trypan blue, and Wright's stain, viable cell counts and morphologic identifications were done. Cells were kept (4°) for virus isolation and titration. Spleens were cut into several pieces with a scalpel and sterile suspensions of these cells were prepared as had been done with cells derived from thymuses. Suspensions of bone marrow cells were obtained from femurs by forcing sterile RPMI 1640 through the marrow cavity with a syringe and 22-gauge needle. Cell clumps and debris were removed with a 26-gauge needle.

Cells from spleens and bone marrows were washed, suspended in 5 ml of RPMI 1640 medium, and layered carefully onto 3 ml of Hypaque–Ficoll. Again, using a 14% concentration of Ficoll, mononuclear cells were separated by centrifugation as previously described for peripheral blood. Samples of the separated cells were taken with Pasteur pipets, washed, resuspended, and retained (4°) for Wright's staining and quantitative virus culture. Adherent cells from mononuclear cell suspensions derived from the spleen were removed as previously described for peripheral blood. These cells were also kept (4°) for staining and virus cultures. At stain, both adherent and nonadherent fractions contained >95% lymphocytes, as in peripheral blood.

Separations of nonadherent leukocytes by reactions with specific antibodies. The following procedures with nonadherent mononuclear suspensions obtained from the spleen and peripheral blood of 14-day-old Swiss mice were used in order to subdivide the cells into thymocyte antigen and light chain antibody-bearing fractions. Nonadherent cell suspensions from the spleen and peripheral blood were divided into two 0.5-ml aliquots each containing approximately 1×10^6 cells.

Five-tenths milliliter of each suspension was mixed with an equal volume of a 1:128 dilution of rabbit anti-mouse thymocyte serum (Microbiological Associates, Bethesda, Md.) in RPMI 1640. Then 1 ml of 1:3 dilution of guinea pig complement (Colorado Serum, Denver, Colo.) was added and the entire mixture was allowed to incubate for 90 min at 37°.

In all reactions with antithymocyte serum, it was depleted of complement by absorption with Noble agar for 30 min at 4° (Difco Laboratories, Detroit, Mich.) and stored as a 1:3 dilution in RPMI at −70° (6). A 1:3 dilution of antithymocyte serum when added to guinea pig complement (see above) in preliminary *in vitro* cytotoxicity tests lysed 98 to 100, 37 to 40, and 2% of suspensions of thymocytes, nonadherent spleen, and bone marrow cells, respectively (8).

The remaining aliquots of nonadherent spleen and peripheral blood leukocytes were incubated with equal volumes of a combined 1:8 dilution of rabbit anti-mouse κ light chain and rabbit anti-mouse λ light chain serum (Miles Laboratories, Elkhart, Ind.) for 45 min at 22°. One milliliter of absorbed guinea pig complement was added, and the incubation was continued for another 45 min at 37°. This antiserum mixture lysed 58 to 65% of nonadherent leukocytes derived from bone marrow or spleen. The spleen cells used here had previously been treated with antithymocyte serum as de-

scribed. Combined anti-light chain serum also lysed 10 to 15% of the cells in a suspension of thymocytes. Thus, elimination of antibody-bearing cells was neither as complete nor as specific as that of thymocytes (8, 9).

After final incubations, cells were centrifuged (400*g*, 4°, 10 min), washed (1×), and resuspended in 2 ml of RPMI 1640. Dead cells were removed by centrifugation with Isopaque–Ficoll containing 0.1% sodium azide (10). Live cells were again washed, resuspended, and sampled for viability (trypan blue stain) and purity (Wright's stain). The remaining cells were kept (4°) for quantitative virus cultures.

Results. Experimental infections. Times of appearance of interferon and neutralizing antibody in serum during coxsackievirus B3 infection in 14-day-old Swiss mice have been studied in this model (2). In earlier studies serum interferon appeared 24 hr after infection and 48 hr later neutralizing antibody was present (11).

Infected nude mice appeared well throughout and plasma interferon was present on Day 1 (mean titer, 1/100), peaked on Day 2 (mean titer, 1/150), and declined thereafter. Likewise, neutralizing antibody was detected in the plasma of Nude mice on Day 3 (mean titer, 1/48), peaked on Day 6 (mean titer, 1/210), and declined thereafter (Fig. 1). Although neutralizing antibody has been shown to persist in Swiss mice infected with coxsackievirus A9 (12), it was not present after Day 20 in Nu/Nu mice here.

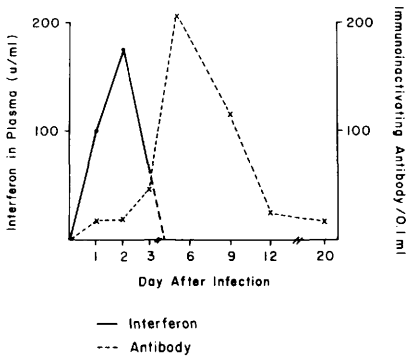

FIG. 1. Interferon and immunoinactivating antibody in plasma of Nude mice infected with coxsackievirus B3.

Total white blood and absolute mononuclear cell counts in peripheral blood during infection. In Swiss mice total white blood cells were significantly decreased from those of noninfected controls on Days 2 and 3 ($P < 0.05$, t test) but not later. The reason for the apparently elevated total and absolute mononuclear cell counts in control mice on Days 1 and 2 is not entirely clear, but the results were repeated twice. Total white blood cell counts in infected and control Nu/Nu mice were similar on Days 2, 3, and 4 ($P > 0.05$, t test, Figs. 2 and 3). In Nu/Nu mice absolute mononuclear cell decrements were significantly lower than those in the controls on Days 2, 3, and 4 ($P < 0.05$, t test).

Viremia in Swiss and Nu/Nu mice. In experiments using Swiss mice coxsackievirus B3 was found in pooled plasma on Days 1 to 5 (Fig. 4). Mean titers not indicated in Fig. 4 were 10^2 (Day 1), $10^{3.8}$ (Day 2), $10^{2.8}$ (Day 3), 10^2 (Day 4), and 10^1 TCD_{50}/ml (Day 5), respectively. In Nude mice, virus was found in plasma on Days 1 (mean titer, 10^3), 2 (mean titer, $10^{4.8}$), and 3 (mean titer, $10^{3.5}$ TCD_{50}/ml). In both Swiss and Nu/Nu mice the Hypaque–Ficoll separated–washed red blood cells and polymorphonuclear leukocytes were free of virus on each of Days 1 through 6. The mononuclear cell fraction in this experiment from peripheral blood of infected Swiss mice, like red blood

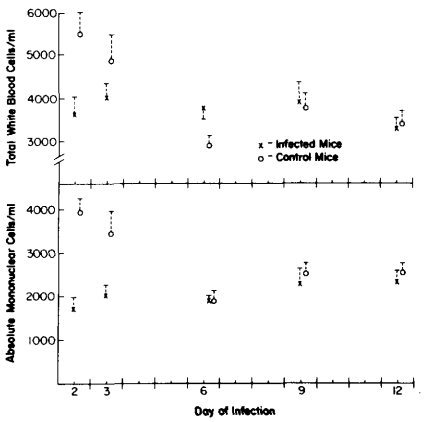

FIG. 2. Total white blood and absolute mononuclear cell counts in 14-day-old Swiss mice infected with coxsackievirus B3. The bracketed lines indicate the standard error of the means.

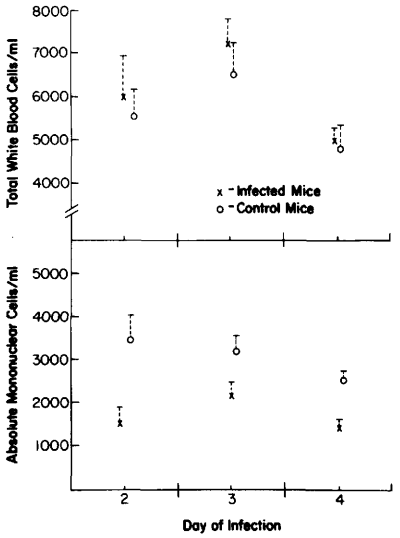

FIG. 3. Total white blood and absolute mononuclear cell counts in adult Nu/Nu mice infected with coxsackievirus B3. The bracketed lines indicate the standard error of the means.

cells and polymorphonuclear leukocytes, had no virus on Days 1, 2, 4, 5, or 6. Mononuclear cell suspensions from Swiss mice were positive for coxsackievirus B3 on Day 3.

In an experiment to determine whether thymus or marrow-derived mononuclear leukocytes contained coxsackievirus B3 during the passage of virus in peripheral blood, the same experiment was performed using athymic Nu/Nu mice. Virus was found in the plasma on the first 3 days of infection; erythrocytes and polymorphonuclear leukocytes remained sterile throughout, but the separated mononuclear leukocytes of one of three experiments were positive on the third day of infection (Fig. 4).

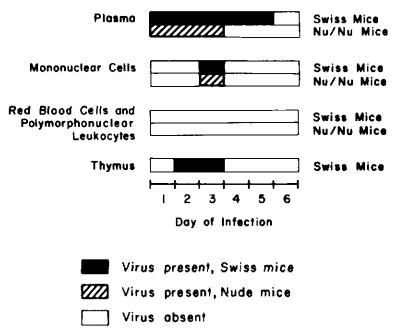

FIG. 4. Distribution and duration of coxsackievirus B3 in blood of infected Swiss and Nude mice.

Thymuses from Swiss mice contained high titers of virus on Day 2 (mean, $10^{3.5}$ TCD_{50}/g) and 3 (mean titer, $10^{6.0}$ TCD_{50}/g) but this organ contained no virus earlier or later. Titrations were done using exsanguinated mice and thoroughly washed tissues. On Day 3, the titer of virus in the thymus exceeded that in pooled plasma, and coxsackievirus seems to have multiplied at this site. Nude mice had no recognizable thymus at autopsy.

Coxsackievirus B3 was found in the heart (Day 4, mean titer, $10^{2.8}$ TCD_{50}/g) and pancreas (Day 3, mean titer, 10^{6} TCD_{50}/g) of Nu/Nu mice. Coxsackievirus B3 also multiplies in the hearts, skeletal muscle, and pancreas of Swiss mice (2). The latter findings have been reported.

Quantitative coxsackievirus B3 titers in separated suspensions of mononuclear leukocytes from peripheral blood, thymus, spleen, and bone marrow. Virus-bearing mononuclear cells in blood during coxsackievirus B3 infection in 14-day-old infected Swiss mice were studied on Days 2, 3, and 4 (Table I). This experiment was repeated in full twice. Data were similar, and the results of the first experiment are reported.

In this experiment, on the second day of infection whole blood (10^{4} TCD_{50}/ml), plasma ($10^{2.5}$ TCD_{50}/ml), and the mononuclear cells contained coxsackievirus B3. Red blood cells and polymorphonuclear leukocytes, as before, were virus-free. Suspensions of mononuclear cells from the thymus (10^{4} TCD_{50}/ml), bone marrow ($10^{2.5}$ TCD_{50}/ml), and spleen also had virus. After treatment with mouse antithymocyte serum plus complement, nonadherent cells from peripheral blood contained no virus. After similar incubations with anti-κ and anti-λ light chain serum, remaining nonadherent cells had $10^{2.0}$ TCD_{50}/ml. These results suggest that on Day 2 of infection the coxsackievirus B3-positive mononuclear cells in peripheral blood may include Ig^{-} leukocytes. However, nonadherent cells from the spleen were virus-positive after both incubations with antithymocyte and anti-light

TABLE I. DISTRIBUTION OF COXSACKIEVIRUS B3 IN BLOOD, BONE MARROW, THYMOCYTES, AND SPLEEN IN INFECTED 14-DAY-OLD SWISS ICR MICE

Body fluid, cell, or tissue	Coxsackievirus B3 (log TCD_{50}/ml) Day of Infection 2	3	4
A. Peripheral blood			
1. Whole blood	4.0	0	0
2. Plasma	2.5	0	0
3. Mononuclear cells			
(a) Adherent cells	2.5	2.5	1.5
(b) Nonadherent cells			
(1) After ATS[a] (Ig^-)	0	2.0	2.0
(2) After ALS[a] (Ig^-)	2.0	2.5	2.5
4. Red blood cells with polymorphonuclear leukocytes	0	ND*	ND
B. Bone marrow	2.5	0	0
C. Thymocytes	4.0	0	0
D. Spleen			
1. Adherent cells	3.0	2.5	0
2. Nonadherent cells			
(a) After ATS	3.0	2.5	2.5
(b) After ALS	2.5	3.0	3.0

[a] ATS, anti-thymocyte serum; ALS, anti-light chain serum; ND, not done. This experiment was repeated twice with similar results. The results of the first experiment are reported.

chain mouse sera indicating that Ig^+ leukocytes also may be virus-positive.

In this same experiment on the third and fourth days of infection, viremia in whole blood had ceased, and plasma, bone marrow, and thymocytes no longer retained coxsackievirus B3. However, on these same days, cells from peripheral blood, adherent cells, and Ig^+ and Ig^- mononuclear suspensions were also virus-positive.

Nonadherent leukocytes (Ig^+ and Ig^-) from spleen, like the same populations from peripheral blood, also retained virus on Days 3 and 4 (Table I). Adherent cells derived from the spleen were positive on Days 2 and 3, but negative on Day 4.

Discussion. These experiments report circulating infectious virus-positive mononuclear leukocytes during viremia of coxsackievirus B3 infection. The virus–mononuclear cell complexes were found in both 14-day-old Swiss mice and in young adult athymic mice. Coxsackievirus B3 positive mononuclear leukocytes persisted in blood in two experiments with Swiss mice after virus was no longer present in plasma. On the third and fourth days of infection, concentrations of specific anti-coxsackievirus B3 antibodies in plasma had risen, thus neutralizing virus free in plasma, but apparently had not yet covered all antigen-binding sites upon the surface of circulating leukocytes. On the second day of infection, studies with antithymocyte serum suggested that the circulating infectious mononuclear cells might be derived from the thymus. Erythrocytes and polymorphonuclear leukocytes were free of virus throughout.

In Swiss and athymic mice virus multiplied in several murine organs, including heart, pancreas, and striated muscle. Plasma viremia ceased when neutralizing antibody was present in excess (12, 13). Athymic mice produced anti-coxsackievirus B3-neutralizing antibody which was no longer present on Day 20. This early antibody is probably IgM and not thymus-dependent.

In both Swiss and Nude mice lymphocytopenia occurred on the first several days of infection, a finding which has been noted many times in other virus infections (14). Mononuclear cell migration to the liver, spleen, and bone marrow has been shown in mice with Newcastle disease

virus infection. This migration may account for the lymphocytopenia of many virus infections including the coxsackievirus B3 infections in Swiss and athymic mice used here. Such migration has not been shown for coxsackievirus infections to date.

Perhaps the virus-positive mononuclear leukocytes, which unfortunately, cannot be further defined by the methods used here may be more readily removed from the circulation by the mononuclear phagocytic systems of the liver or spleen, and the virus-positive lymphocytes which migrate to the liver and spleen could be an adaptive mechanism for survival.

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Received March 19, 1980. P.S.E.B.M. 1980, Vol. 165.