technique which simplified procedures considerably.

Each of the sera from the patients with rheumatoid arthritis was inactivated at 56°C for 30 minutes and absorbed twice with packed sheep erythrocytes to remove low titered heterophile agglutinins. With the immune rabbit sera, specificity was checked beginning at a dilution of 1:200 without preliminary absorption. We were not concerned with heterophile agglutinins because all sera were from highly immunized animals and could, therefore, be tested beyond the normal range for this antibody. We did, however, check 2 sera, an anti-M and an anti-ovalbumin beginning at a titer of 1:4 and continuing to 1:1028. Neither direct hemagglutination nor abnormal settling patterns occurred. With normal mouse and guinea pig serum, however, titers as high as 1:128 were obtained against tannic acid treated and formalinized sheep erythrocytes alone and also with similarly prepared cells to which 1% BSA had been coupled. With these and with some selected rabbit antisera, preliminary absorption with sheep erythrocytes may be necessary.

Summary. A simple method has been described which allows firm coupling of a variety of proteins to tanned sheep erythrocytes in the presence of formalin. Cells, so treated, agglutinate strongly and specifically with antibody; they also form suitable settling patterns and maintain specific reactivity and sensitivity for a full 2 weeks. Agglutination with immune rabbit sera with as little as 0.04 μg of antibody protein per ml serum was readily detected. The tannic acid-formaldehyde (TAF) test compared favorably with 2 other established hemagglutination procedures for detecting rheumatoid factor in 10 sera from patients with rheumatoid arthritis.

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Suppression of Interferon and Antibody and Multiplication of Newcastle Disease Virus in Cytomegalovirus Infected Mice.* (31740)

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Studies of mice infected with mouse cytomegalovirus (MCMV) showed that during the first 2 weeks of infection, animals were markedly inhibited in their capacity to produce circulating interferon in response to intravenous injection of Newcastle disease virus (NDV)(1). This finding suggested that NDV might be handled differently by MCMV-infected mice.

NDV penetrates mouse cells(2) and is a potent stimulator of mouse interferon(3,4). However, it does not normally multiply in mouse tissue. Ginsberg(5) showed that large quantities of NDV administered intranasally produced a toxic pulmonary consolidation in Swiss white mice. Inactivated virus did not induce the pathology, and although infectious virus was necessary, it was rapidly cleared

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from the lung and multiplication did not occur. Liu and Bang(6) described both pneumonitis and a late-appearing encephalitis in certain inbred strains of mice after intranasal or intracerebral NDV, but again the pathogenesis did not involve multiplication of the virus.

NDV autointerference occurs in calf kidney cell culture, and is mediated by interferon (7,8). Whether the interferon stimulated by NDV in mouse cells plays a role in aborting the infection in that species is not known. This MCMV-induced suppression of interferon response to NDV in mice provided a dual-infection model for studying the role of interferon in mouse host response to NDV.

In the investigations to be described, it was shown that whereas a suppression of interferon response to NDV in MCMV-infected mice occurred, there was a normal interferon response to endotoxin. Furthermore, in such MCMV-infected mice, NDV multiplied in the spleen during the first 72 hours following intravenous inoculation; but in most animals this extraordinary infection was abruptly terminated by 96 hours. Studies of clearance and antibody response to NDV revealed significant alterations of both mechanisms in MCMV-infected animals. Administration of exogenous interferon to MCMV-infected animals resulted in a significant reduction of NDV multiplication.

Materials and methods. Mice: Swiss white mice (CD-1) were used in all experiments; similar results were found in both 10-12 g weanlings and 25-30 g adults. All MCMV infections were initiated by intraperitoneal (IP) injection of $10^{5.2}$ plaque-forming units (pfu) of MCMV per mouse.

Cell cultures and viruses: Methods of quantitation of MCMV and interferon were identical with those previously described(1); secondary cell cultures of trypsinized mouse embryo tissue (METC) with 10% starch overlay(9) using medium 199 were employed for all plaque assays. Primary chick embryo cell cultures (CECC) were prepared from 9-day chick embryos by trypsinization and grown in 60 mm plastic plates in medium 199 and horse serum. NDV of the CG_9 strain, originally obtained from Drs. Marie Foard and Frederick Bang, was passed 2 times in 11-day chick embryos and harvested after 24 hours' incubation at 37°C. The infected allantoic fluid was pooled and stored in vials at −50°C. This material had a titer of $10^{8.2}$ pfu per 0.2 ml. Tissues to be tested for NDV were prepared as 10% suspensions, clarified by light centrifugation and stored at −50°C or 4°C until plaque assayed for NDV on CECC.

Endotoxin: Endotoxin extracted from *E. coli* 0113 by the Boivin procedure(10) was kindly supplied by Dr. A. Braude. It was stored at 4°C as a dry powder until dissolved in 0.85% saline in a concentration of 250 μg per 0.2 ml immediately prior to use.

Interferon production and assay: Mouse serum interferon was induced with NDV according to the method of Baron and Buckler (3), and with endotoxin according to the method of Stinebring and Youngner(11). Interferon assay was performed using encephalomyocarditis virus (EMC) as an indicator of interferon activity. The titer of interferon was defined as the reciprocal of that dilution of serum which caused a 50% reduction in control plaque count of 60-120 pfu of EMC in METC.

Neutralization: Sera to be tested for anti-NDV neutralizing activity were inactivated at 56°C for 30 minutes; serial 2-fold dilutions were then incubated with 30-60 pfu of NDV at 36°C for one hour, and tested for NDV by plaque assay in CECC. The neutralizing antibody titer of a serum is herein defined as the reciprocal of the highest dilution which reduced control plaque counts by more than 50%.

Experimental procedures and results. Demonstration of interferon response to endotoxin in MCMV-infected mice: Mice were inoculated IP with MCMV; controls received diluent. On the 4th or 5th day of infection one group of animals was given *E. coli* 0113 endotoxin, 250 μg per mouse intravenously (IV), and these mice were exsanguinated 2 hours later. The remaining mice were given NDV that day, $10^{8.2}$ pfu IV, and exsanguinated 5 hours later. Sera were stored at 4°C until assayed for interferon.

Table I shows the interferon titers of the various sera. There was no difference be-

TABLE I. Comparison of Circulating Interferon Response of MCMV-Infected Mice to Endotoxin or NDV IV.

Group	Interferon titer in serum	
	2 hr after IV endotoxin, 250 μg *E. coli* 0113	5 hr after IV NDV (10^8 pfu)
MCMV-infected*	80,†‡ 160, 160, 320	<5, <5, <5, 20
Control	80, 320, 320, 320	80, 160, 160, 160

* Mice injected with interferon stimulus on Day 4 or 5 of MCMV infection.
† Interferon titer expressed as the reciprocal of the highest serum dilution causing a 50% reduction of 60-120 pfu EMC in METC.
‡ Each figure represents pooled sera from 5-10 animals.

tween the responses of infected and control mice to intravenous endotoxin, whereas MCMV infected animals failed to respond with circulating interferon 5 hours after IV NDV.

Fate of infectious NDV administered intravenously to MCMV-infected mice: Experimental animals were given MCMV and controls received diluent. Four days later, all animals in both groups were given NDV, $10^{8.2}$ pfu IV. Subsequently, groups of 2-5 mice were sacrificed at 5, 24, 48, 72 and 96 hours and determinations were made of the NDV titer of blood, spleen, lung and liver. In addition, 5-hour sera were tested for interferon, and 48- and 96-hour sera were tested for anti-NDV activity. Some animals were tested at 10 days for NDV neutralizing antibody.

Fig. 1 shows the concentration of NDV pfu in spleens at various intervals. Spleens from control animals showed a steady decrease in NDV titer, and infectious NDV was virtually eliminated by 48 hours. Viremia was seen in only one group of control mice after 5 hours. In contrast, the titer of NDV in spleens of MCMV-infected animals failed to drop at 24 hours and showed a definite increase at 48 and 72 hours, differing at those times by as much as 4 logs from control spleen titers. Low grade NDV viremia was a frequent occurrence at 24 and 48 hours and in one group was found as late as 72 hours; the occurrence of viremia correlated with a high NDV titer in the spleen. The multiplication of NDV terminated spontaneously before 96 hours.

Fig. 2 shows the same data in terms of mean spleen titer at each interval, and in addition, mean NDV pfu concentration in lung and liver are shown. While the spleen is clearly the primary site of involvement, there is a definite delay in elimination of NDV from the lungs of MCMV-infected mice. The liver is apparently the most efficient of the 3 organs in inactivating or eliminating NDV in both groups; the small amount of NDV occasionally recovered from the livers of dually infected mice can be correlated with occurrence and titer of viremia in the same group of animals.

Studies of the sera of these MCMV-infected mice revealed the same marked inhibition of interferon response 5 hours after IV NDV as had been shown before. Control animals had interferon titers ranging from 80-320 units per 0.25 ml of serum.

Impairment of neutralizing antibody responses: Plaque-reduction neutralization tests showed no difference between the sera of dually infected and control animals at 48 hours. However, at 96 hours there was slightly less anti-NDV neutralizing antibody in the sera of MCMV-infected mice, and by 10 days this difference was pronounced, as shown in Table II. While control animals had titers of greater than 40, experimental sera caused 50% neutralization of NDV only at 1:5 or 1:10 dilution.

Clearance of NDV: Fig. 3 shows the results of studies to determine the rate of disappearance of circulating NDV during the first 5 hours after IV injection. Twenty minutes after injection of $10^{8.2}$ pfu, the average titer had dropped more than 3 logs in both MCMV-infected and control animals. At each time interval studied the mean NDV pfu concentration was greater in the blood of MCMV-infected animals than in that of controls. The difference between means at 5 hours was significant at the 0.05 level.

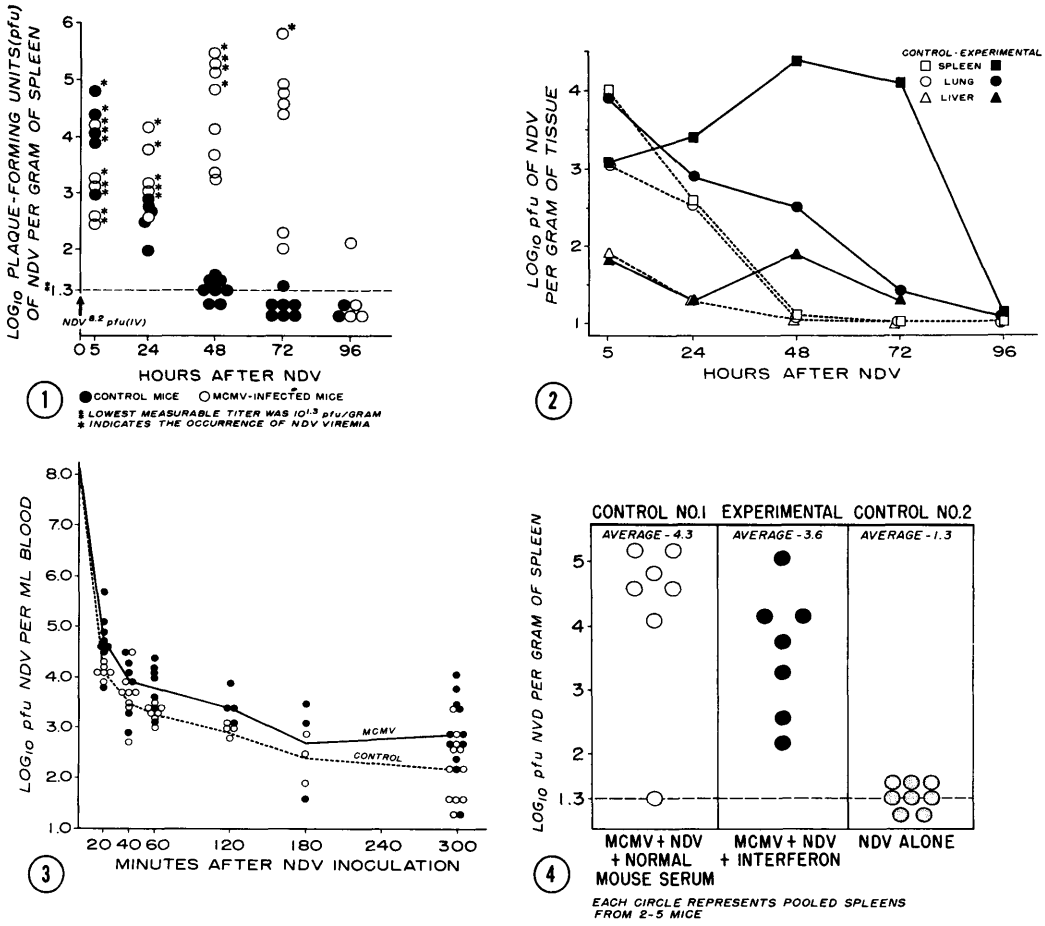

FIG. 1. Titer of infectious NDV in mouse spleen at intervals after intravenous injection of $10^{8.2}$ pfu of NDV. Each point represents result of testing spleens of 2-5 mice.

FIG. 2. Mean titer of NDV in spleen, lung and liver after intravenous injection of $10^{8.2}$ pfu NDV: MCMV-infected (closed symbols) and control mice (open symbols).

FIG. 3. Clearance of NDV after intravenous injection of $10^{8.2}$ pfu NDV in MCMV-infected (dots) and control (open circle) mice.

FIG. 4. Effect of administering exogenous interferon on titer of NDV in mouse spleens 48 hr after NDV, $10^{8.2}$ pfu (IV).

TABLE II. Suppression of NDV Neutralizing Antibody Response in MCMV-Infected Mice.

Source of serum		
MCMV-infected mice 10 days after NDV (IV)	Control mice 10 days after NDV (IV)	Normal uninoculated mice
*5, 5, 10, 10	>40, >40, >40, 80, 80, 80	<5, <5, <5

* Each figure is result of testing pool of serum collected from mice and is the reciprocal of highest serum dilution which reduced control NDV plaque count by more than 50%.

Absence of NDV multiplication after intranasal inoculation: When NDV, $10^{7.8}$ pfu per mouse, was administered intranasally after light ether anaesthesia and the lungs were assayed for NDV at 48 and 72 hours, it was found that NDV was eliminated rapidly and with equal efficiency by both groups of mice, MCMC-infected and control animals.

Effect of exogenous interferon on NDV replication: A pool of mouse serum interferon was prepared from normal mice by exsanguination 5 hours after administration

of NDV intravenously. Normal serum was obtained from uninoculated litter mates. Mice in the fourth day of their MCMV infection were then divided into 2 groups: half were given 320 u serum interferon IP 1 hour prior to intravenous NDV, $10^{8.2}$ pfu, and these animals received an additional 640 u interferon IP 2 hours after NDV. The remaining mice were given equal volumes of normal mouse serum before and after NDV. Fig. 4 shows the NDV titers in spleens of these 2 groups and in normal mice 48 hours after IV NDV. When the aberrant datum (1,3) in Control Group No. 1 is omitted as an outlier, the lowering of NDV titer in the spleens of those animals receiving interferon was significant, P being less than 0.01.

Discussion and conclusions. NDV does not normally replicate in mice. Thus these findings represent a synergistic effect of MCMV and NDV infections with the result that NDV's host range was broadened. Examination of several possible contributory mechanisms revealed, in addition to the previously demonstrated suppression of interferon response, that neutralizing antibody response and clearance of NDV from the blood were impaired.

Henry Wagner *et al*(12) found that systemic viral infections impaired the capacity of the reticuloendothelial system to clear colloidal particles, (in contrast to bacterial infections which enhanced RE clearance). In this regard it is of interest that the reticuloendothelial organs are the site of maximum involvement in the early stages of MCMV infection(13). Brunner *et al*(14) studied the fate of P32-labeled NDV in mice during the first 20 minutes after IV injection. They found the liver cleared more than 50% of the NDV, and the lung and spleen cleared only 6% and 2% respectively. When the RE system was blockaded with thorotrast, NDV clearance was markedly impaired. The spleen and lung were less affected by the blockade and cleared proportionately more than before. Brunner's finding that the liver was the primary site for clearing NDV particles is an apparent contrast with the results reported here, but in his studies infectious NDV was not measured.

Wheelock has shown that Friend virus (FV) alters the interferon response of mice to Sendai virus in much the same way as does MCMV(15). It was not determined whether Sendai virus would multiply to a greater extent in that system, however.

The results of several studies lend precedent to the possibility that MCMV-induced interferon inhibition played an important role in establishing conditions for NDV multiplication. Interferon mediated autointerference of NDV has been demonstrated in cell culture (7,8). Vesicular stomatitis virus inhibits NDV induced interferon production in Krebs-2 cells, but in this system NDV does not multiply even in the absence of interferon (16). A recent report described enhanced multiplication of NDV in HeLa carrier cultures of Sendai virus, and implicated interferon as the determinant factor(17). Postic showed that interferon inhibition by actinomycin D in mice resulted in enhanced Sindbis virus infection(18). In our studies the capacity of exogenous interferon to significantly lower the NDV titer in spleens of MCMV-infected mice supports this hypothesis.

Other examples of virus synergism have been studied. These were demonstrated *in vitro,* however, and the responsible mechanisms were not well defined. They include mumps and Sindbis(19), hog cholera and NDV(20), and rabies and lymphocytic choriomeningitis viruses(21). The latter two systems clearly do not depend on interferon and further elucidation of these synergistic relationships may be pertinent to the MCMV-NDV model.

Failure of MCMV-infected mice to produce a normal neutralizing antibody response to NDV is particularly striking in view of the fact that they were exposed to greater quantities of NDV for a longer period (as a result of NDV multiplication) than were the control mice. Depression of antibody responses in animals infected with viruses has been described previously. Peterson *et al* have shown that antibody response to T_2 coliphage was reduced in mice infected with Gross' passage A leukemia virus(22), and Deut *et al* have shown that in these same mice allograft

rejection capacity is reduced(23). Salaman and Wedderburn confirmed earlier work by Old and Clark and demonstrated that infective Friend virus (FV) depressed the primary hemagglutinin response to sheep red blood cells in mice(24,25). Their data indicate that the effect was correlated directly with the dose of virus and temporally with the maximum enlargement of the spleen. Although the spleen was not the only organ involved in the mediation of this response (FV reduced the immune response of splenectomized mice), FV infection reduced the number of antibody producing cells in the spleen as revealed by Jerne's technique. Siegel and Morton have shown that infection with another leukemogenic murine virus, Rauscher virus (RV), suppresses the immune response to bovine serum albumin(26). They postulate that the suppression occurs as a result of virus infection of pluripotential stem cells in the spleen. Gross has reported evidence which suggests that Friend and Rauscher viruses may be the same(27), the similar properties noted above are consistent with this view. Glasgow(28) has shown that the mouse leukocyte plays a significant role in interferon response and resistance to vaccinia, and it is possible that depressed leukocytes or leukocyte precursors might be the factor common to the synthesis of both interferon and antibody. Our studies also would suggest that the spleen is the residence of the responsible cell type and experiments employing fluoresceen labeled antibody are being carried out to determine which cells are infected with MCMV and NDV. Thus two viruses FV and MCMV inhibit the interferon response and FV, RV and MCMV inhibit the antibody response in mice. All severely affect the spleen. FV and RV are leukemogenic; MCMV does not seem to be(29).

The mechanism responsible for this MCMV-induced inhibition of synthesis of two dissimilar proteins (interferon and NDV antibody) must be relatively specific; the preformed endotoxin-induced interferon response is normal in MCMV-infected animals, and infective NDV is synthesized. It may be relevant that Ho and Kono(30) have shown that actinomycin D exerts a similar differential inhibition in rabbits.

Finally, it is of interest that despite the impaired host defenses described, NDV replication in the spleen is spontaneously terminated before 96 hours. The mechanisms responsible for this termination are at present as obscure as are those responsible for the inhibition of the antibody and interferon responses.

Summary. MCMV inhibition of interferon response in mice was found to be specific for virus-induced interferon; endotoxin evoked a normal interferon response. NDV multiplied in the spleens of MCMV-infected mice during the first 72 hours after intravenous inoculation, a period when interferon response was suppressed. In these animals, in addition to the inhibition of interferon response, there was significant impairment of clearance and a suppression of NDV neutralizing antibody production. The relative role each plays in the multiplication of NDV in MCMV-infected mice remains undefined. Extensive MCMV replication in the spleen and significant pathologic alteration of it indicate that that organ is central to these effects. Despite these alterations of host response, NDV infection spontaneously terminated at 96 hours. At a stage during MCMV infection when interferon synthesis was suppressed, NDV multiplication was partially inhibited by administering exogenous interferon.

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Liver Manganese in Hemochromatosis. (31741)

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Hemochromatosis is a disease in which occur excessive absorption of iron and cirrhosis of the liver(1). The increased liver iron has been suggested as the cause of the cirrhosis(1,2,4). But the failure to produce cirrhosis with iron loading in animal experiments (1,13,14) and observations of transfusional iron loading of the liver without cirrhosis (1,5,6) suggest that this may not be the case.

It has recently been shown that manganese absorption in rats increases when iron absorption is increased by bleeding or dietary deprivation of iron(12). The possibility is apparent that in diseases in which iron absorption is increased manganese absorption may also be increased. Since liver cirrhosis may be produced in experimental animals by injecting manganese into the peritoneum(7) the hypothesis was considered that absorption and deposition of manganese and iron in the hemochromatotic liver is the cause of the cirrhosis.

If manganese absorption is increased in hemochromatosis then the concentration of manganese in the liver might be increased, that organ being the principal storage site (8,9) for manganese.

The concentration of manganese in liver samples from patients dying with hemochromatosis was compared with the manganese concentration in liver samples of patients dying of myocardial infarction.

Comparative concentrations were determined by neutron activation analysis. Formalin-preserved samples were studied. Two groups of hepatic samples were collected. Twenty cases of "hemochromatosis" were studied. All met these criteria:

(1) Males over 35 years of age
(2) Liver cirrhosis
(3) Liver hemosiderosis
(4) Iron deposition in other tissues of the body
(5) No history of multiple transfusions
(6) No history of excess iron ingestion

Fig. 1 shows a photomicrograph of typical