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Received June 14, 1967. P.S.E.B.M., 1967, v126.

Interferon Production by Germfree Mice.* (32413)

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Since the initial studies of interferon by Isaacs and Lindenmann(1), ample evidence has been accumulated which has suggested that interferon plays a significant role in host-resistance to primary viral infections(2-4). Germfree animals have played important roles in investigations which otherwise would have been complicated by the presence in them of a microbial flora(5). Because bacteria and bacterial products have been shown to induce the formation of interferon-like substances *in vivo*(6), it seemed likely that the germfree animal might again provide a more refined test animal for investigations involving the induction and production of interferon *in vivo*. Mice respond to intravenous inoculation of Newcastle Disease virus (NDV) with high levels of serum interferon (7). Therefore, the same system was employed to determine the general capacity of germfree mice to produce interferon in response to viral stimulation.

Materials and methods. Animals. Germfree CFW mice were reared and maintained by the routine procedures developed at Lobund Laboratory for germfree animals(8-10). The conventional control mice were derived originally from germfree stock which had been adapted to the microflora of the animal quarters. The mice were 5 weeks of age and consisted of approximately equal numbers of males and females.

Viruses. The Asian strain of Newcastle Disease virus (NDV) was propagated in embryonated chicken eggs and demonstrated a titer of $10^{9.7}$ PFU/ml. A large plaque variant of encephalomyocarditis virus (EMC-r) was kindly provided by K. Takemoto of National Institutes of Health, Bethesda, Md. Vesicular Stomatitis virus (VSV) was obtained through the courtesy of B. Postic, Graduate School of Public Health, University of Pittsburgh, Pa. The latter 2 viruses were propagated in MCN mouse fibroblast cell cultures and were used for assay of interferon.

Tissue cultures. A continuous mouse fibroblast cell line(11), designated as MCN cells, kindly provided by F. Dienhardt, Presbyterian-St. Luke's Hospital, Chicago, Ill., was used for virus propagations and titrations, and for assay of interferon. Primary cell cultures were prepared from trypsinized chicken embryos and used for NDV titrations. All cultures were grown in Eagle's Minimum Essential Medium (MEM) with non-essential amino acids and glutamine added, and supplemented with 15% heat-inactivated calf serum. Penicillin and streptomycin were included at concentrations of 100 units/ml and 0.1 mg/ml, respectively. Sodium bicarbonate was added to give a concentration of 0.04%. The plaque medium recipe and the procedures for plaque assays were those described in a personal communication from B. Casto, Graduate School of Public Health, University of Pittsburgh, Pa.

Interferon production. Interferon was induced by intravenous inoculation of mice with NDV *via* the lateral tail veins. The blood was collected by incision of the axillary blood vessels, and stored overnight at 4°C. Spleens and livers were placed in vials and immediately frozen in an alcohol-dry ice bath.

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They were then stored in a mechanical freezer at below −50°C.

Interferon preparation. The serum was separated from the blood clot by centrifugation. The organs were thawed and homogenized in 10 or 20% suspensions (w/v) in Eagle's MEM with a Ten-Broeck glass homogenizer. The cells were then disrupted by freezing and thawing 3 times or by sonication treatment for 2 minutes at 13.5 amp in an MSE ultrasonic power unit. To eliminate cell receptors, each preparation was then centrifuged at 100,000 × *g* for one hour in a Spinco Model L ultracentrifuge. Residual NDV was eliminated from all preparations by acid treatment at pH 2 for one to three days at 4°C.

Interferon induction in germfree mice. Within one hour of removal from germfree isolators, groups of germfree mice were inoculated with NDV, and the blood and organs were collected at various times thereafter up to a period of 24 hours. Therefore, the animals described in this report as "germfree" may be interpreted as ex-germfree mice. It was believed, however, that the response exhibited by these ex-germfree mice would be essentially identical to that of technically germfree mice, since the period of exposure would be so short: less than 24 hours and in most cases, 6 hours or less. In fact, experimental evidence has supported this assumption. Two groups of germfree CFW mice, each consisting of at least 5 animals, were inoculated intravenously with NDV. One group was inoculated within the isolator and maintained germfree for the next 5 hours, at which time they were removed from the isolator and exsanguinated immediately. The other group was inoculated after removal from the isolator and maintained in a clean environment for the same length of time, then their blood was collected. There was no detectable difference in the serum interferon levels of these 2 groups of mice.

Interferon titration. A plaque-inhibition assay system was chosen to assay the interferon preparations, using EMC or VSV as the indicator viruses. A constant dose of virus was added to a series of MCN monolayer cultures in 30 ml Falcon flasks. Immediately after, a constant volume of either 2- or 4-fold dilutions of the interferon preparation was added to 3 replicate cultures. Controls consisted of cultures inoculated with diluent alone and of cultures inoculated with only virus and diluent. A standard interferon preparation was always assayed together with the test materials. Following inoculation, the cultures were maintained at 37°C for one hour to permit adsorption of virus. The cultures were then covered with an agar overlay containing Eagle's MEM with non-essential amino acids and glutamine, and with 0.1% bovine serum albumin, penicillin (100 units/ml), streptomycin (0.1 mg/ml), and sodium bicarbonate (0.04%). Plaques were read at 48 hours after adsorption of virus. The measure of interferon activity was the dose which depressed the plaque counts to 50% of the controls, for which Lindenmann and Gifford have suggested the abbreviation PDD_{50} or 50% plaque depressing dose(12). Equidistant dose-response curves were required for valid comparisons of interferon samples tested simultaneously.

Indentification of interferon. The interferon preparations studied have been tested and found to possess the following properties of interferon: cell species specificity, insensitivity to antibody against the inducer, non-sedimentability at 100,000 × *g* for one hour, inability to inactivate virus directly, inhibitory activity against heterologous virus, trypsin sensitivity, insensitivity to nucleases, relative heat stability, and insensitivity to acid.

Results. Serum baseline inhibitory activity. Groups of 4 to 10 germfree mice and of conventional mice were bled, and the pooled sera were tested for baseline virus inhibitory activity against the formation of plaques by either EMC or VSV. These animals were either uninoculated or were inoculated with NDV-free allantoic fluids. Ten serum pools were tested: 4 from germfree mice and 6 from conventional mice. No plaque inhibition equal to or greater than 50% was observed when the serum was diluted 1/4 or more. In 2 of 3 sera tested at a dilution of 1/2, the conventional mouse serum was inhibitory, while in the one instance of testing undiluted serum, both the germfree mouse

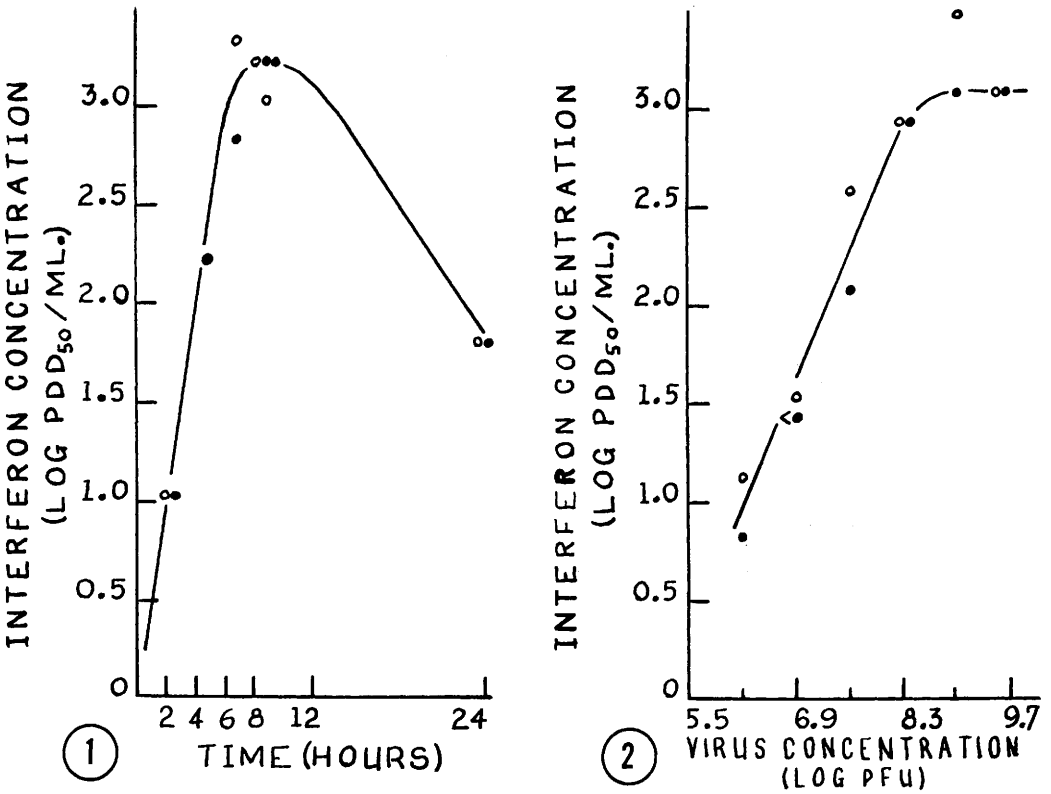

FIG. 1. Serum interferon responses of germfree and of conventional CFW mice at various times after intravenous inoculation with $10^{9.7}$ PFU or NDV. Each point represents the average response of 4 animals. Open circles (○), germfree animal responses; closed circles (●), conventional animal responses.

FIG. 2. Serum interferon responses of germfree and of conventional CFW mice 6 hr after intravenous inoculation with various doses of NDV. The symbol (<) means that response was less than that indicated, but lower responses were not tested for. Open circles (○), germfree animal responses; closed circles (●), conventional animal responses.

serum and the conventional mouse serum demonstrated a definite inhibitory activity. The results seem to indicate that the baseline inhibitory activities of germfree and of conventional mouse sera were of low magnitude and, in general, similar.

Serum interferon levels with time. Germfree and conventional CFW mice were inoculated intravenously with undiluted stock NDV. The average serum interferon responses of 4 germfree mice and of 4 conventional mice were measured at various intervals thereafter (Fig. 1). The optimum interferon response to this dose of NDV occurred at about 6 hours after inoculation. The time pattern of serum interferon response to NDV stimulation in germfree animals seemed to be essentially the same as that in the conventional animals.

Serum interferon levels related to dose of NDV. Groups of 4 germfree CFW mice and groups of 4 conventional CFW mice were inoculated with 0.2 ml of 5-fold dilutions of stock NDV. At 6 hours post inoculation serum interferon levels were determined (Fig. 2). There was what appeared to be a linear increase in interferon production with increasing doses of NDV at least up to a dose of about 10^9 PFU. Above 10^9 PFU there appeared to be a plateau. The germfree mice again appeared to respond to various doses of NDV with serum interferon levels of the same order of magnitude as those of conventional mice.

The effect of monocontamination. Germfree mice were monocontaminated *per os* with either *Escherichia coli* or *Lactobacillus casei*. The 4-hour serum interferon response

to an optimum dose of NDV at 1, 3 and seven days after monocontamination was determined in germfree and in conventional control mice. The average interferon response of 5 mice was measured on each occasion (Table I). The cecums, livers, lungs, and spleens of the gnotobiotes were tested qualitatively for the presence of the bacterial contaminants: in every instance the cecums were positive while the other organs tested were negative. The 2-fold decrease in the serum interferon level on the seventh day after exposure to *E. coli* and the 4-fold decrease on the third day after exposure to *L. casei* may reflect the normal variability of the interferon response of groups of mice of the size studied. Thus, we have been unable to demonstrate that monocontamination had any effect on the ability of germfree mice to respond to NDV with high levels of serum interferon.

TABLE I. Effect of Monocontamination of Germ-Free CFW Mice on Serum Interferon Response to NDV.

Animals, treatment*	Interferon titer (PDD_{50}/ml) Day 1	Day 3	Day 7
Conventional, *E. coli*	200	200	200
Gnotobiotes, *E. coli*	400	400	200
" , *L. casei*	400	100	400
Conventional, none	200, 200		
Germfree, none	400, 200		

* Groups of 5 animals each, monocontaminated *per os*, then inoculated intravenously 1, 3 or 7 days later with $10^{9.1}$ PFU NDV. Serum interferon levels 4 hr after inoculation with NDV were then measured.

TABLE II. Spleen and Liver Interferon Levels in Germfree CFW Mice and in Conventional CFW Mice After Intravenous Injection of NDV.

Tissue	Time (hr)	Animals*	Interferon titer (PDD_{50}/100 mg)
Spleen	2	Uninoculated controls	6.6
		Conventional	6.6
		Germfree	53
	6	Conventional	210
		"	270
		Germfree	850
		"	1070
Liver	6	Conventional	27
		Germfree	106

* Groups of 4 animals each. Except for the uninoculated control animals, all animals were inoculated intravenously with $10^{9.7}$ PFU NDV.

Interferon levels in spleens and livers. Groups of germfree and of conventional mice, consisting of 4 animals each, were tested for spleen and liver interferon levels at 2 and at 6 hours after intravenous inoculation with undiluted stock NDV (Table II). The levels of interferon in the spleens and livers of the germfree mice were at least equal to, if not greater, than those of the conventional mice.

Discussion. A preliminary study (unpublished) indicated that germfree C3H mice responded to NDV stimulation with serum interferon production which was at least 2-fold higher than that in conventional counterpart C3H mice. It would seem now, however, that the differences observed among the C3H mice were probably due to the range of variability of the response in groups of mice of the numbers studied. In the present study, 2- and 4-fold differences in serum interferon levels between germfree and conventional groups of mice have been observed (4 to 5 mice per group), but the overall response with time and to various doses of NDV seemed to be essentially the same for both germfree and conventional CFW mice.

Studies of the baseline inhibitory activity of sera from untreated control germfree and control conventional mice showed no differences. If there is a difference it would not be beyond the range of normal variability of the interferon response inherent in groups of mice of the size studied. Methods more refined than those used in this study might be required to make them apparent.

Finally, the levels of interferon in the spleens and livers of the germfree mice were at least equal to, if not greater, than those of the conventional mice. These results are in agreement with those DeSomer and Billiau obtained when spleen interferon levels of germfree rats were measured 2 hours after inoculation with heat-killed *E. coli* and compared with the responses of conventional rats to a similar inoculum(13). It is possible that the difference in the spleen and liver interferon levels observed between the germ-

free and conventional mice may reflect no more than the normal variability of response in such small groups of animals. Nevertheless, two facts from the present study suggest that the higher levels of spleen and liver interferon among germfree mice may be significant: (a) the tissue interferon levels were consistently higher among germfree mice whether tested at 2 hours or at 6 hours and whether tested in spleens or in livers, and (b) repetition of the test gave the same results: higher interferon levels in the spleen extracts from germfree mice.

The enhanced interferon response among the germfree mice may be due to higher storage levels of preformed interferon or of interferon precursors(14) because of their comparatively "pristine" condition. If so, the germfree animal may represent the optimum tool for demonstrating the existence of such entities.

Summary. A study was made to determine the relative capacity of germfree mice to respond to virus stimulation with interferon production *in vivo*. Germfree CFW mice responded to intravenously inoculated Newcastle disease virus with a pattern of serum interferon production similar to that of conventional mice. Moreover, the baseline inhibitory activity of sera from unstimulated control germfree and conventional mice were the same. However, there was some indication that the spleen and liver interferon responses of germfree mice may be higher than those of conventional mice. It is suggested that such higher responses in germfree mice might be due to higher storage levels of preformed interferon or of interferon precursors because of their comparatively "pristine" condition.

This investigation was supported in part by USPHS Training Grant GM 1248 from Nat. Inst. Health and in part by a grant from Allen County Cancer Society.

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Received June 15, 1967. P.S.E.B.M., 1967, v126.

Magnesium Deficiency and Urea Cycle Enzymes in Rat Liver.* (32414)

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Induction of magnesium deficiency in the rat has been observed to produce several metabolic abnormalities. Recently we have reported a generalized aminoaciduria in the presence of an increase in the activity of sodium-potassium activated kidney adenosine triphosphatase, and the suggestion was made that the alteration in the urinary amino acid pattern was the result of renal tubular dysfunction rather than an "overflow of aminoaciduria"(1). Since protein metabolism was altered in the deficient animals, it seemed appropriate to study the influence of magnesium deficiency on the urea cycle enzymes. These enzymes have been shown to undergo adaptative changes whenever dietary protein

* Supported in part by USPHS Grant NBO 3152.
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