

tion occurred by the morning of day 5, but not by day 4, after the last injection. They considered from ovulation-blocking experiments that the time of LH release in this case was rather different from that of 4-day cyclic rats, *i.e.*, the LH release began about 2 PM but lasted longer. Subsequently most rats ovulated between about midnight and 5:30 AM. In addition, they claimed that the vaginal smear was not a reliable indicator of the day of ovulation. The apparent discrepancies between the results of the present experiment and theirs might be due to differences in the kind and dosage of steroids employed, and the route of steroid administration. Our results would appear to show by contrast that (i) the vaginal smear is reliable in predicting the day of ovulation, (ii) time of ovulation in rats treated orally with CAP and then withdrawn is similar to normal cyclic rats and (iii) ovulation occurred by day 4 after withdrawal from treatment.

Summary. After oral treatment with 10 mg chlormadinone acetate (CAP) for 5 days, the time of ovulation was examined by the detection of tubal ova in rats which came back into vaginal proestrus on day 3 after with-

drawal (66% of treated rats). This was compared with that of normal cyclic rats. In both control and treated rats, some animals had started ovulating at 1 AM, and nearly all of the rats had ovulated by 2 AM. The number of ova increased from 2 to 6 AM. From the evidence obtained it can be said that in CAP-treated rats the time of ovulation did not differ from that of the normal cyclic (4 days) rats, *i.e.*, the timing of ovulation occurred in the same relation to the diurnal rhythm of lighting.

The authors are grateful to Professor S. Takeuchi for interest and encouragement during the experiment. Thanks are due to Dr. Neena B. Schwartz, University of Illinois, for her kind criticism of their results and arrangement to submit their paper.

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Received August 30, 1967. P.S.E.B.M., 1967, v126.

Immunoglobulin Classes of Serum Neutralizing Antibody Formed in Response to Immunization with Dead Influenza Virus Vaccine. (32597)

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The immunological basis for protection against influenza virus infection is complex. Protection does not entirely correlate with serum antibody level(1) and seems to be higher after infection with live virus than after dead virus immunization(2). The present study was undertaken to determine, in humans, which class of immunoglobulins possessed

neutralizing antibody activity in serum after parenteral immunization with dead influenza virus vaccine. This was done to establish whether this difference in protection could be explained on the basis of different classes of immunoglobulins.

Materials and methods. Serum specimens were obtained before and for 4 weeks after the initial dose of vaccine from 6 normal adult male volunteers hospitalized at the National Institutes of Health. Each volunteer received 2 weekly subcutaneous injections of 1 cc of the commercially available killed influenza

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TABLE I. Mean Immunoglobulin Concentration in Sephadex G-200 Pools.

	"M pool"	"A pool"	"G pool"
IgM	0.49*	0	0
IgA	0.02	0.71	0.72
IgG	0.07	0.50	4.10

* mg/ml

virus vaccine.† The composition of the vaccine was 100 CCA units of A/PR8, A1/Ann Arbor/1/57, A2/Japan/170/62 and A2/Taiwan/1/64 and 200 CCA units of B/Maryland/1/59.

Gel filtration. The fractionation of serum was carried out using Sephadex G-200.§ The effluent from each serum specimen was partitioned so that 3 fractions were obtained, each containing predominantly either IgM, IgA or IgG(3).

Immunoglobulin assay. IgM, IgA and IgG concentrations were determined by the radial diffusion precipitin method in agar(4).

Immunoglobulin absorption. IgM, IgA and IgG were individually absorbed out of selected pools by adding in small increments specific antisera until no further precipitate could be detected(5).

Hemadsorption-inhibition-neutralization test. Influenza virus antibody levels were determined by the standard neutralization test(6). Specimens of serum and of eluate pools were tested against 30 to 300 50% tissue culture infectious doses of A2/Bethesda/10/63 influenza virus.

Results. Characterization of eluate pools. As previously mentioned, the eluate from each serum specimen obtained after Sephadex G-200 filtration was fractionated into 3 pools, each containing predominantly IgM, IgA or IgG. Table I shows the mean immunoglobulin concentration in each pool. As can be seen, the "M pool" was very specific and had a mean IgM content of 0.49 mg/ml. However, it was impossible to clearly separate IgA from IgG. The "G pool" contained almost 10 times more IgG than the "A pool" but had as much IgA. The "A pool" had a mean IgA content of 0.71 mg/ml, but also had a mean IgG content of 0.50 mg/ml.

Antibody response. The mean serum antibody response is shown in Fig. 1. The mean preinoculation titer was 1:54 with a range of 1:32 to 1:128. The response was essentially similar in all volunteers and is well represented by the mean values. The maximum rise occurred in the 1st week and peak titers were obtained by day 14 and persisted for at least 28 days. The titers in the "G pools" closely paralleled those in whole serum whereas titers in the "M pools" were extremely low (maximum mean titer of 1:2). The titers in the "A pools" were intermediate, and when individual pools were examined, it became apparent that the levels of antibody in the "A pool" were closely correlated to the amount of IgG contamination in these pools. A similar correlation was found in the "M pools;" when IgG was absent, there was no antibody activity and when antibody was present, IgG could be found in the "M pools."

Absorption studies. The impression that the neutralizing antibody was present only in the IgG class of immunoglobulin was further studied by absorption tests. "M pools" and "A pools" with relatively high antibody titers were selected and IgG and either IgM or IgA were absorbed with specific antisera. Rep-

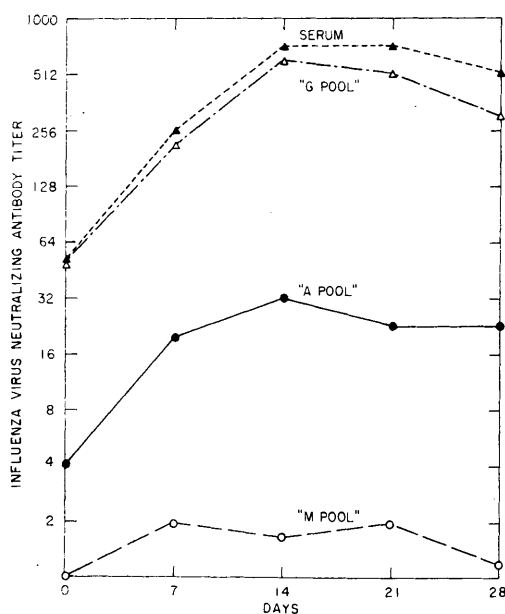


FIG. 1. Influenza virus neutralizing antibody titers in whole serum and unabsorbed Sephadex G-200 eluate pools.

† Winthrop Laboratories, New York, New York.

§ Pharmacia Fine Chemicals, Uppsala, Sweden.

TABLE II. Immunoglobulin Levels and Antibody Titers in Sephadex G-200 Pools Before and After Absorption with Specific Antisera.

Specimen	Absorbed with	Immunoglobulin level		Neutralizing antibody titer	
		M	A		
19472 M pool		.44*	<.07	.31	1:32
19472 M pool	Anti IgM	—†	<.07	.33	1:32
19472 M pool	Anti IgG	.43	<.07	—	1:<2
19901 A pool		—	1.0	.4	1:32
19901 A pool	Anti IgA	—	—	.31	1:32
19901 A pool	Anti IgG	—	1.02	—	1:1.5

* mg/ml

† Not detectable

representative examples for an "M pool" and an "A pool" are shown in Table II. When IgM was removed from an "M pool," the antibody did not change; however, removal of the IgG caused a greater than 16-fold reduction in antibody titer. Similarly, when IgA was removed from an "A pool," the antibody did not change; however, removal of the IgG again caused a greater than 16-fold reduction in antibody titer.

Discussion. The results of the present study indicate that following parenteral immunization with dead influenza virus, the neutralizing antibody formed belongs solely to the IgG class of immunoglobulin. Previous work has shown that the same statement is true of the antibody formed after experimental infection with an A2 influenza virus administered *via* the upper respiratory tract(7). In neither case could the experiments be done with volunteers absolutely lacking preinoculation neutralizing antibody to influenza virus. It is possible that after 1st exposure to influenza viral antigen IgM antibody is formed.

Our interest has been directed at trying to understand the immunological basis of protection against influenza virus infections. This protection is not always directly related to levels of serum antibody(1). Previous work in this laboratory has shown that both homotypically and heterotypically acquired antibody against influenza virus is found in the IgG class of the immunoglobulin(7). This and the findings in the present study indicate that the reason for the lack of complete correlation between serum antibody levels and protection does not result in a difference in the classes of immunoglobulins.

It has also been shown that although natu-

ral infection is a more potent inducer of respiratory secretion antibody, parenteral immunization with dead virus causes a rise in this secretory antibody(8). The neutralizing antibody in secretions belongs to the IgA class of immunoglobulins. Our data, which show that there is no serum IgA antibody, add further support to the concept that secretory antibody is locally produced.

Summary. Serum specimens obtained at weekly intervals from 4 normal adult volunteers immunized with dead influenza virus vaccine were fractionated on Sephadex G-200 in order to study the classes of immunoglobulins which make up the influenza virus neutralizing antibody. Neutralizing antibody activity was found only in the IgG class of immunoglobulins. This finding is similar to the results obtained in volunteers inoculated with live influenza virus *via* the respiratory tract. Since the neutralizing antibody in respiratory secretion is mostly found in the IgA class, the lack of IgA serum antibody supports the hypothesis of local production of antibody in external secretions.

We wish to express our gratitude to Dr. Sheldon M. Wolff for allowing us to use normal volunteers under his care.

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Received August 30, 1967. P.S.E.B.M., 1967, v126.

The Blood and Bone Marrow Neutrophil Response to Graded Doses of Endotoxin in Mice.* (32598)

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A change in neutrophil concentration in venous blood can be effected by a change in the rate at which neutrophils are released to the blood from the marrow, by a change in the rate of outflow of neutrophils from the blood, by a change in the proportion of neutrophils in blood which are margined along vessel walls rather than circulating freely, or by any combination of these 3 mechanisms.

It has been suggested that endotoxin administration is associated with an increase in the rate at which neutrophils are released from the marrow to the blood(1,2), that it influences the proportion of neutrophils which are margined along vessel walls(3,4) and also effects the rate at which neutrophils leave the blood(5).

That the effect of endotoxin upon neutrophil kinetics is somewhat complex is suggested by studies in which the response of the blood neutrophil concentration has been compared after different doses of endotoxin. Neutropenia is the 1st change observed after the injection of endotoxin in dogs(6) and the degree and duration of the neutropenia increase as the dose of endotoxin is increased. After relatively small doses of endotoxin, neutropenia is followed by neutrophilia and the height of the neutrophilia increases as the dose of endotoxin is increased. However, Sheagren *et al*(7), who

did not follow counts beyond 5 hours, failed to observe neutrophilia in the monkey after very large doses of endotoxin had been given. Neutropenia was not observed after small doses in the rabbit(8,9) or monkey(7). A clear relationship of neutrophil changes to the dose of endotoxin administered also was not observed in the mouse(10) or in man(11,12).

With the development of technics for determining the total number of neutrophils in the marrow of a mouse(2,13), it is now possible to correlate changes occurring in blood and marrow neutrophils simultaneously. Such correlations were used in the present study in an attempt to more definitively characterize the effect of endotoxin upon neutrophil kinetics. For this purpose, mice were injected with increasing amounts of endotoxin and the effect of endotoxin upon the total number of neutrophils in the marrow and their concentration in blood was determined at various times thereafter.

Materials and methods. Female mice (C57B1 ♀ × DBA ♂)F₁, bred in our laboratory from parent stock purchased from Jackson Laboratory, weighing 18-22 grams and approximately 8 weeks of age were used in all experiments. Mice were housed 8-10 per cage and Purina Lab Chow and water were available *ad libitum*. Animals were injected intraperitoneally with either 0.01 μg, 0.1 μg, 0.2 μg, 5.0 μg, or 25.0 μg of *S. typhosa* endotoxin (Lipopolysaccharide B-0901, Difco Laboratories) in a volume of 0.1 cc isotonic saline.

Blood for leukocyte counts was collected from the orbital sinus in heparinized micro-

* This investigation was supported by a research grant (AM-04489) and a graduate training grant (2A-5098) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland.

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