INFECTION AND IMMUNITY, Apr. 1978, p. 115-119
0019-9567/78/0020-0115$02.00/0

Vol. 20, No. 1
Printed in U.S.A.

Antibody Production and Protection Against Influenza Virus in Immunodeficient Mice

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Received for publication 20 October 1977

The roles of T and B cells in the immune response to influenza virus were studied by using mice deficient in either T cells (athymic nude) or immunoglobulin production (CBA/N). The serological responses of these mice to either whole or disrupted A/Aichi/2/68 influenza virus vaccines were examined, and the protective effect of these inoculations was tested by challenge infection with mouse-adapted A/Aichi/2/68 influenza virus. In contrast to normal mice, neither strain of immunodeficient mouse produced detectable serum antibody after inoculation with either type of vaccine. CBA/N mice immunized with intact virus vaccine were protected, however, against subsequent lethal challenge. CBA/N mice inoculated with disrupted virus vaccine and nude mice inoculated with either disrupted or whole virus vaccine were not protected against viral challenge. Evidence of immunological memory was observed in CBA/N and nude mice that had survived live virus challenge after immunization with inactivated vaccine.

The persistence of live influenza virus in the lungs, spleens, or brains of athymic (nude) mice following respiratory challenge indicates the need for functional thymocytes for the elimination of virus and recovery from infection by the host (24, 28). It is also known that purified influenza hemagglutinin, whole influenza virus, and live infectious virus are thymus (T)-dependent antigens and that nude mice or thymectomized, lethally irradiated, and bone marrow-reconstituted mice do not develop serum antibody after inoculation or infection with these preparations (5, 24, 27). Whether this defect in antibody production is responsible for the observed persistence of influenza virus in nude mice is not clear.

We have utilized nude mice, which lack T cells (8, 18), and CBA/N mice, which manifest a genetically determined deficient serological response to most T-independent antigens (2), to study the respective roles of T and B cells both in the development of antibody after immunization with Formalin-inactivated influenza vaccines and in subsequent protection from lethal infection. Responses to both intact and chemically disrupted influenza virus vaccines were examined, since these two types of vaccine have been shown to induce different responses in unprimed animals (4, 17) and humans (3, 19).

(This material is from a thesis presented by S. L. to the Department of Microbiology of the George Washington University in partial fulfillment of the requirements for the M.S. degree.)

MATERIALS AND METHODS

Mice. Male nude (nu/nu) mice [N:NIH(S)] bred on an NIH Swiss background (9), Swiss (+/nu) mice, and CBA/N mice were obtained from the Rodent and Rabbit Section, National Institutes of Health, Bethesda, Md. Male CBA/CaJ mice were obtained from Jackson Laboratories, Bar Harbor, Me. Nude and Swiss mice were 8 to 10 weeks of age and were maintained under specific-pathogen-free conditions until the onset of the experiment. CBA/N and CBA/CaJ mice were 6 to 8 weeks of age.

Vaccines. Both influenza virus vaccines used were Formalin inactivated. Whole virus vaccine (Eli Lilly & Co., Indianapolis, Ind.) was zonally purified, whereas the subunit vaccine (Wyeth Laboratories, Inc., Marietta, Pa.) was chromatographically purified and disrupted with tri(n)-butyl phosphate and Polysorbate 80. Both met Food and Drug Administration requirements for safety and potency and contained 700 chick cell-agglutinating units of A/Aichi/2/68 (H3N2) and 300 chick cell-agglutinating units of B/Mass/1/71 influenza virus antigens per 0.5-ml dose.

Virus. A/Aichi/2/68 virus used in challenge studies was mouse adapted by five passages in outbred NIH Swiss mice (6) and stored at −70°C. This virus pool had a 50% mouse lethal dose of $10^{1.5}$ when administered as an aerosol as described previously (24) and contained $10^{9.1}$ 50% egg infectious doses (EID_{50}) per ml.

Vaccination and challenge. Mice were vaccinated intraperitoneally with 0.5 ml of a 1:10 dilution of uninfected allantoic fluid, whole influenza virus vaccine (A/Aichi/2/68), or tri(n)-butyl phosphate-disrupted influenza virus vaccine (A/Aichi/2/68). Twenty-eight days after immunization, serum samples were collected either by sacrificing some of the mice and bleeding from the axillary vein or by bleeding all

mice from the orbital sinus. Groups of 40 to 60 mice were challenged the following day with varying doses of mouse-adapted A/Aichi/2/68 influenza virus in an aerosol chamber as previously described (24). Control mice were exposed to uninfected allantoic fluid. Twenty-one days after challenge, mice were sacrificed and examined for serum antibody and for the presence of virus in tissues.

Virus isolation. Lung, spleen, and brain specimens were removed aseptically and prepared for virus isolation in embryonated hens' eggs by homogenization with a Potter tissue grinder in 2 ml of beef heart infusion broth containing amphotericin B (0.3 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). Allantoic fluids were titrated for virus by hemagglutination of chick erythrocytes in microtitration plates (23). Representative samples of viral isolates were characterized as A/Aichi/2/68 by hemagglutination-inhibition (HAI) tests with specific antisera.

Serological testing. Individual serum samples were treated with *Vibrio cholerae* receptor-destroying enzymes, heat inactivated for 30 min at 56°C, and absorbed with chick erythrocytes (26). HAI antibody titers using 4 hemagglutinating units of homotypic whole virus antigen were determined in microtitration plates (23).

Statistical analysis. Post-vaccination and postchallenge antibody titers of mice were compared by the multivariant *t* test (11). Mortality after challenge was compared by the chi-square test with Yates correction.

RESULTS

Response in B lymphocyte-deficient mice. CBA/N mice did not produce detectable HAI antibody after vaccination (Table 1), although antibody was produced in immunologically normal (CBA/CaJ) mice. Most mice were resistant to a challenge dose of $10^{5.5}$ EID_{50}. At the higher challenge dose of virus, approximately 80% (16/19 and 15/19) of the normal mice survived. Of the CBA/N mice receiving intact virus vaccine, 75% also survived challenge, whereas protection was not observed in recipients of disrupted vaccine.

Virus was not detected in the lungs, spleens, or brains of surviving CBA/CaJ or CBA/N mice.

Response in T lymphocyte-deficient mice. In the immunologically intact +/nu animals (Table 2), all immunized mice developed detectable HAI antibody (although +/nu mice receiving disrupted vaccine had a lower mean antibody titer than did those receiving whole virus vaccine) and were protected from both doses of live virus challenge. The mean antibody titer (1:891 and 1:630, not shown on table) of +/nu mice that received disrupted vaccine and survived subsequent live virus challenge was significantly greater ($P < 0.01$) than the mean antibody titer (1:294 and 1:239) of +/nu challenged survivors that had received intact vaccine. Vaccination of nude mice failed to induce either detectable HAI antibody or resistance to the challenge virus. The mean time of death of the nu/nu virus controls was significantly greater ($P < 0.05$) than that of the +/nu virus controls challenged with $10^{5.5}$ EID_{50} of virus. This delayed mean time of death was not observed at the higher challenge dose nor in the immunized group. The unusually high mortality of the nude mice serving as controls at the $10^{5.5}$

TABLE 1. *Antibody production, mortality, and serum HAI antibody titers of CBA/CaJ and CBA/N mice after vaccination and challenge with A/Aichi/2/68 (H3N2) influenza virus*

Strain	Immunization (day 0)[a]	Mean HAI antibody titer 28 days after vaccination	Challenge (day 29)	Mortality[a] after virus challenge:		Mean HAI antibody titer 21 days after virus challenge:	
				$10^{5.5}$ EID_{50}	$10^{6.5}$ EID_{50}	$10^{5.5}$ EID_{50}	$10^{6.5}$ EID_{50}
CBA/CaJ	Control	<1:8	Control	0/8	0/10	<1:8	<1:8
	Control	<1:8	V[c]	3/28	23/30	1:80	1:37
	Intact	1:30	V	1/19	3/19	1:222	1:549[d]
	Disrupted	1:35	V	0/19	4/19	1:181	1:981[d]
CBA/N	Control	<1:8	Control	0/7	0/10	<1:8	<1:8
	Control	<1:8	V	3/28	19/30	1:24	1:194
	Intact	<1:8	V	0/19	5/20[e]	1:315[d]	1:630[d]
	Disrupted	<1:8	V	2/19	19/20[e]	1:256[d]	1:1024[d]

[a] Control, Uninfected allantoic fluid; Intact, 70 chick cell-agglutinating units of whole A/Aichi/2/68 virus vaccine intraperitoneally; Disrupted, 70 chick cell-agglutinating units of tri(*n*)-butyl phosphate-disrupted A/Aichi/2/68 virus vaccine intraperitoneally.

[b] Mortality, Number dead per number challenged.

[c] V, Mouse-adapted A/Aichi/2/68 influenza virus.

[d] HAI titers significantly greater than in control nonvaccinated animals after challenge infection ($P < 0.05$, Student's *t* test).

[e] $P < 0.001$.

TABLE 2. *Antibody production and mortality in heterozygous and nude mice after vaccination with A/Aichi/2/68 (H3N2) influenza virus*

Strain N:NIH(S)	Immunization (day 0)[a]	Mean HAI antibody titer 28 days after vaccination	Challenge (day 29)	Mortality[b] after virus challenge[c]:	
				$10^{5.5}$ EID_{50}	$10^{6.5}$ EID_{50}
+/nu	Control	<1:8	Control	0/10	0/10
	Control	<1:8	V[d]	15/28 (10.3)[e]	14/30 (8)[f]
	Intact	1:52[g]	V	0/19	0/20
	Disrupted	1:8[g]	V	0/19	0/20
nu/nu	Control	<1:8	Control	5/10	0/10
	Control	<1:8	V	19/20 (12.8)[e]	24/24 (6.8)[f]
	Intact	<1:8	V	13/13 (11.8)	13/16 (9.5)
	Disrupted	<1:8	V	10/10 (10.8)	16/16 (6.5)

[a] Control, Uninfected allantoic fluid; Intact, 70 chick cell-agglutinating units of whole A/Aichi/2/68 virus vaccine intraperitoneally; Disrupted, 70 chick cell-agglutinating units of tri(n)-butyl phosphate-disrupted A/Aichi/2/68 virus vaccine intraperitoneally.

[b] Mortality, Number dead per number challenged.

[c] Parentheses indicate mean time of death in days.

[d] V, Mouse-adapted A/Aichi/2/68 influenza virus.

[e] $P < 0.05$.

[f] Not significant.

[g] $P < 0.001$.

EID_{50} dosage level (5/10 dead by 21 days after challenge with uninfected allantoic fluid) was attributed to "wasting" disease, which is common among nude mice living outside of specific-pathogen-free areas (8).

A low level of HAI antibody (1:8) was demonstrated in the serum of three nude mice surviving 21 days after challenge with $10^{6.5}$ EID_{50} of virus. Despite the presence of this low level of antibody, approximately $10^{6.3}$ EID_{50} of influenza virus per ml was isolated from the lungs of these animals. No virus was isolated from brains or spleens of nude mice or from lungs, spleens, or brains of normal mice.

DISCUSSION

This study has shown that mice deficient in either T or B lymphocytes did not produce detectable antibody after a dose of inactivated influenza virus vaccine that consistently induced antibody in normal control mice. Despite this absence of detectable antibody, mice with diminished numbers of B lymphocytes were protected from subsequent challenge with live virus and produced antibody after this challenge. The absence of detectable antibody after immunization with influenza virus vaccine may be the result of defective immunoglobulin M production by CBA/N mice (1), coupled with the quantitative reduction in numbers of B cells (22). Alternatively, the CBA/N mice could have a diminished subpopulation of B cells that responds to T-dependent antigens (14).

In contrast to the nude mice, however, CBA/N mice were protected from challenge, and survivors demonstrated an antibody response greater than that observed in mice that had not been immunized before challenge. This indicates that the immunized CBA/N mice were "primed" to respond to a secondary antigenic stimulus (the live virus challenge), even though there was no detectable antibody response after the primary antigenic stimulus. A similar response has been reported in ferrets (16). Virelizier et al. (27) have shown that B-cell memory to these antigens is T independent, even for T-dependent antigens. Since the CBA/N mice develop detectable antibody after challenge, they are therefore not lacking the subpopulation of B cells necessary for memory.

Protection from influenza virus infection correlates with the level of circulating serum antibody in mice (6, 7, 15) and humans (12). Virelizier et al. (27) reported that thymectomized, lethally irradiated, and bone marrow-reconstituted mice could not produce antibody to purified hemagglutinin antigens. Additional studies in nude mice (5, 24) confirmed that production of antibodies against either whole influenza virus or against live infectious virus is T dependent. Our own observations agree with these reports, because nude mice did not develop detectable levels of HAI antibody after vaccination with formaldehyde-inactivated influenza virus vaccines and were not protected.

Although neither strain of immunodeficient mouse produced detectable antibody after a primary exposure to antigen (i.e., by vaccination), low titers of antibody were detected in nude mice that survived a secondary antigenic stimulation (i.e., challenge infection). These three nude mice that survived the challenge of $10^{6.5}$

EID_{50} of influenza virus may indicate that the memory for antibody production is T independent; similar conclusions were reached by previous workers (27). The low titers of antibody, however, may indicate that, in the absence of T cells, the memory B cells are unable to fully express their memory potential. Definite conclusions on this point must await further studies, since certain viral infections have been reported to induce T-cell markers and function on the T-cell precursor of nude mice (21).

Persistence of virus was demonstrated in the lungs of nude, but not CBA/N, mice 21 days after infection with mouse-adapted influenza virus. Persistence of influenza virus in lungs, spleens, or brains has been observed up to 14 days after infection of nude mice with live influenza virus (24, 28). We have recently observed persistent infection in nude mice up to 11 weeks after infection with a live non-mouse-adapted H3N2 influenza virus (D. W. Barry and S. J. Lucas, unpublished data). Although it appears that T cells are required to rid the mouse of infectious influenza virus, further studies are in progress to determine whether persistence of virus might be secondary to a deficient humoral or cellular response after initial viral replication.

T cells may also play a detrimental role in the virus-infected host. We found, as did Sullivan et al. (24) and Wyde et al. (28), that mice with functioning T cells died sooner at one dosage level than those without functional T cells (Table 2). Our findings are consistent with previous reports in which not all challenge doses result in delayed mean time of death (13, 24, 25). This phenomenon may be similar to that seen during lymphocytic choriomeningitis infection in mice, where nonimmunocompetent mice (neonates and immunosuppressed) do not die from acute infection, but normal animals do (10, 20). Additional reconstitution experiments in nude mice should resolve the role of cell-mediated immunity in recovery from a chronic virus infection and the possible harmful role it may play during acute infection.

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