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THE EFFECT OF MULTIPLE IMMUNIZATION ON INTERFERON PRODUCTION

Annie R. Beasley, et al

Miami University

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13. ABSTRACT The purpose of this work was the determination of the effects of various immunization regimens on the capacities of lymphocytes to respond to specific and nonspecific stimuli by blastogenesis and interferon production. Healthy young adult volunteers were immunized with single or multiple vaccines, sequential blood samples were obtained, and lymphocytes were cultured with and without stimuli. After appropriate incubation periods, uptake of tridiated thymidine was determined as an indication of blastogenesis and supernate fluids were harvested for interferon assays. Emphasis was necessarily placed upon standardization of test procedures, as the potency of the stimuli and the cellular immunity of the volunteers were unknown. Optimal stimulating dosages of phytohemagglutinin and of mumps and influenza antigens have been determined, and interferon assays are in progress. Since the studies to date are preliminary and the study group was small, no conclusions can be drawn but definitive investigation can now be undertaken.

Standard methods for in vitro stimulation of mouse spleen cell cultures have also been established, allowing detailed studies of this animal model.

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REPORT NUMBER ONE

THE EFFECT OF MULTIPLE IMMUNIZATIONS ON INTERFERON PRODUCTION

ANNUAL SUMMARY REPORT

ANNIE R. BEASLEY and M. MICHAEL SIGEL

January 11, 1974

Supported by

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SUMMARY

The purpose of this work was the determination of the effects of various immunization regimens on the capacities of lymphocy es to respond to specific and nonspecific stimuli by blastogenesis and interferon production. Healthy young adult volunteers were immunized with single or multiple vaccines, sequential blood samples were obtained, and lymphocytes were cultured with and without stimuli. After appropriate incubation periods, uptake of tridiated thymidine was determined as an indication of blastogenesis and supernate fluids were harvested for interferon assays. Emphasis was necessarily placed upon standardization of test procedures, as the potency of the stimuli and the cellular immunity of the volunteers were unknown. Optimal stimulating dosages of phytohemagglutinin and of mumps and influenza antigens have been determined, and interferon assays are in progress. Since the studies to date are preliminary and the study group was small, no conclusions can be drawn but definitive investigations can now be undertaken.

Standard methods for <u>in vitro</u> stimulation of mouse spleen cell cultures have also been established, allowing detailed studies of this animal model.

FOREWORD

In conducting the research described in this report, the investigators have adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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BODY OF REPORT

The Medical Departments of the Armed Forces have long been concerned over the explosive outbreaks of viral respiratory disease in induction centers, with illness essentially confined to recruits. It is possible that the extensive immunization procedures practiced at the time of induction may play a role in these outbreaks by influencing nonspecific defense mechanisms, including the functional activities of lymphocytes. In this respect, it is known that lymphocytes react to appropriate stimuli not only by antibody production but also by blastogenesis and by production of several biologically active factors, including interferon (IF). Conceivably, commitment of the major population of lymphocytes to synthesis of antibodies to a large array of immunogens administered over a short period of time could lead to depletion of cells capable of production of other protective substances, thereby precipitating and/or exacerbating disease. For example, there may develop a deficit in the capacity to elaborate IF. It was our intent to investigate this possibility, using human volunteers and further employing the mouse as a model.

HUMAN VOLUNTEER STUDIES. Fourteen healthy young adult males were selected for the initial studies with single or multiple immunizations with the following vaccines:

a. Yellow fever (4 persons)
b. Bivalent influenza (3 persons)
c. Attenuated mumps (1 person with negative skin test)
d. Mumps, influenza and tetanus toxoid (2 persons)
e. All of the above plus rubella (4 persons)

All vaccines were administered within a period of five days. Prior to immunization, blood samples were taken for initiation of lymphocyte cultures with and without phytohemagglutinin (PHA) and sera were collected to serve as a baseline for subsequent antibody assays.

Sequential bleedings were made on days 2, 9, 12, 19 and 43. For each time period, as for preimmunization, sera were frozen and lymphocyte cultures were initiated with and without PHA and viral antigens. For blastogenesis determinations, 2 μCi of tritiated thymidine was added to cultures 16 hours prior to termination by centrifugation, washing, and precipitation with 5% trichloracetic acid. Cultures with PHA were

terminated after three days incubation, while those with viral antigens were incubated 5 days. Additional unlabeled cultures were terminated at the same time periods and supernatant fluids were harvested for subsequent interferon assays.

The blastogenic stimulation indices (SI) in response to PHA are shown in Table 1. As has been reported by numerous workers, there is seen considerable variation between individuals. It is interesting to note the periodic day-to-day variation in the responsiveness of the cells of some individuals. In other persons, however, there was a fairly uniform pattern of reactivity in which variations are less pronounced. In this latter category are two extremes - MDJ, with a consistently very low response (an average SI of 4.7), and M.D., with a consistently high reactivity (average SI of 105.7).

Only preliminary data are available and the study group is small, but it is of interest to compare the blastogenic responses to PHA of cells of individuals receiving single vaccines (yellow fever, mumps or influenza) with those of cells from persons receiving the combination of immunogens. (This combination, of course, no where approaches the number administered to Armed Forces recruits over a short period of time.) When the SI obtained at two and nine days after the initial injection(s) are compared with the response prior to immunization, there appears to be a slight depression in the responsiveness of cells from those receiving multiple vaccines. Thus in four persons receiving yellow fever only, one showed no change, one exhibited a decrease, one an increase and the fourth an increase then a decrease. Similarly, of the individuals receiving influenza only, one had a decrease in response, one an increase and one an increase then a decrease. Among the four receiving multiple vaccines, there was only a single individual whose lymphocyte response did not change. In the remaining three, a decreased response was observed at either two days or eight days. As we have indicated, the number of individuals is too small to allow formulation of conclusions, but suggest that further studies should be made along this line.

The two specific stimuli selected were mumps skin test antigen (Eli Lilly and Company) and A2/Aichi/2/68 influenza (obtained from Dr. A. Bernstein of Wyeth Laboratories Inc.) in the form of a subunit concentrate containing 12,800

Table 1. PHA-stimulation of Human Lymphocytes Before and After Immunization.

Vaccine(s)	Person	PHA stimulation index, *cultures of day 0	2	9	12	19	43
Yellow fever	JH	75.6	ND	6.8	36.5	70.6	63.7
	MD	7..4	50.6	99.0	104.7	203.0	105.8
	GW	23.0	ND	67.6	ND	17.5	67.4
	JAC	34.2	112.8	14.7	35.3	47.1	24.9
Influenza	RML	29.1	10.1	3.5	13.4	ND	20.2
	RF	11.1	29.5	18.9	55.9	73.1	73.8
	MDB	11.7	5.7	25.7	28.3	22.5	ND
Mumps	ERR	11.1	10.8	48.3	34.6	35.6	44.1
Mumps, influenza tetanus	MDJ	ND	4.3	ND	4.2	7.8	2.7
	SLW	ND	29.0	ND	6.4	55.1	29.7
Mumps, influenza, tetanus, yellow fever, rubella	WL	43.2	59.1	11.0	17.3	34.1	ND
	GES	7.0	11.3	7.0	29.5	22.7	18.6
	KMC	165.6	ND	53.2	46.3	69.8	64.0
	EMC	31.8	11.3	30.1	26.2	46.5	79.1

*Stimulation index = $\frac{\text{average dpm/culture with mitogen}}{\text{average dpm/culture without mitogen}}$

hemagglutinating units/ml. As was to be anticipated, technical problems were encountered in obtaining transformation with these antigens. These problems included choice of basic culture medium, source and concentration of serum for cell culture, methods for preparing cell suspension and dilution of antigen to be employed. These problems were particularly acute with influenza, as we started with subjects of unknown cellular immunity. Further, the test antigen was toxic for the cultured cells at relatively high dilutions - a problem subsequently resolved by dialyzing the antigen.

In spite of these technical difficulties, we have succeeded in demonstrating specific reactivity of lymphocytes to both of these antigens, as measured by DNA synthesis (i.e., increased incorporation of tritiated thymidine). Table 2 shows the blastogenic responses to PHA and influenza antigen of cells cultured approximately six weeks after initiation of the various immunization regimens, and also shows the pre- and post-immunization hemagglutination inhibition antibody titers. Setting an arbitrary figure of 2.2 as the lower limit of a significant SI with influenza antigen, and comparing the results in relation to immunization with influenza vaccine, one can see that four of the five non-immunized individuals had neither an antibody rise nor a positive SI. (The one individual in this group who showed an antibody rise plus a positive SI, a former Marine Corps veteran who is currently in the USMCR, has received many immunizations - including a recent influenza booster inoculation - and therefore may possibly be subject to anamnestic reactions to unrelated antigens.) In contrast, all of the six immunized persons showed an antibody rise and four of these had a positive SI. It is to be noted that the cells of the two immunized individuals with a negative SI by our criterion also responded poorly to PHA.

In evaluating these results, it must be kept in mind that the number of individuals was small. In addition, we do not have baseline SI with viral antigens for these individuals. Now that we have developed the necessary methodology, preimmunization measurements of lymphocyte reactivity to viral antigens will be made in future work.

Table 2. Serum Antibody Titers to Influenza Virus and Transformation of Cultured Lymphocytes by PHA and Influenza.

Immunization	Person	Influenza HI titer serum of day		Stimulation index lymphocytes cultured day 43	
		0	19	$\bar{c}$ PHA	$\bar{c}$ influenza
Yellow fever	JH	1:20	1:20	63.7	1.8
	MD	1:80	1:80	105.8	1.9
	GW	1:40	1:160	67.4	3.1
	JAC	1:80	1:80	24.9	2.0
Influenza	RF	1:20	1:640	73.8	2.3
	RML	1:160	1:640	20.2	1.3
Mumps	ERR	1:40	1:40	44.1	1.7
Mumps, influenza, tetanus, yellow fever, rubella	GES	1:40	1:160	18.6	1.4
	KMC	1:40	1:640	64.0	3.1
	EMC	1:40	1:320	79.1	4.5
	WL	1:40	1:320	34.1	2.4

In a parallel study in this laboratory by J. Lee, a graduate student, it was demonstrated that rubella virus appears to be highly suited for blastogenic studies. In his hands, this virus has given relatively high SI in human cells and in cells of rabbits and fish immunized with rubella virus. In a recent communication, Smith et al. (1) relate similar stimulation of human lymphocytes by this virus. The most important part of Lee's work is that he was successful in inhibiting the blastogenic response of lymphocytes to this virus by specific antiviral antibody. On further study, it was determined that the inhibitory action of his antiserum resided only in the IgM.

More recently, Sigel and Lee showed that virus-antibody complexes may suppress the response of lymphocytes to other mitogens, including PHA. This may have definite relevance to our work in that the multiple vaccines administered to Armed Forces recruits probably engender formation of large amounts of IgM to various antigens. If such IgM should react with the specific antigens, complexes may form which suppress lymphocyte responsiveness to other antigenic stimuli.

As indicated above, sera from each bleeding date were stored for subsequent assay for interferon levels Sequential sera from four individuals who respectively had been immunized with yellow fever, influenza, mumps and multiple vaccines (yellow fever, influenza, mumps, rubella and tetanus toxoid) were screened at dilutions of 1:10 and 1:20 for the presence of circulating IF. For this purpose, monolayer tube cultures of WISH cells were exposed to test preparations and challenged 24 hours later with $10^{2.3}$ ID_{50} of Mengovirus (as determined by parallel titration in the same lot of cells). Cultures were examined daily for viral cytopathic effects (CPE). Interferon titers were expressed as the reciprocal of the dilution of the test preparations which afforded greater than 50 percent protection at the time virus control cultures exhibited advanced CPE. Results of this screening are shown in Table 3. None of the individuals had demonstrable IF in his preimmunization serum, and each showed measurable levels in one or more sera obtained after immunogen administration. Although the results are preliminary, they suggest a variation between individuals - and even in a given person - with respect to time. Further, in some individuals,

Table 3. Preliminary Screening of Human Sera for Interferon Levels Before and After Immunization.

Source of serum		Screen for IF, serum obtained day					
Person	Vaccine(s)	0	2	4	9	12	19
JH	Yellow fever	< 10	≥ 20	≥ 20	< 10	< 10	< 10
MDB	Influenza	< 10	ND	ND	10	< 10	< 10
ERR	Mumps	< 10	< 10	≥ 20	< 10	≥ 20	≥ 20
WL	Yellow fever, mumps, tetanus, influenza, rubella	< 10	ND	≥ 20	< 10	≥ 20	10

there appeared to be an increase in IF levels very early after immunization. These factors may be a reflection of the vaccine(s) administered. These sera will be reassayed by the plaque reduction technic to determine actual IF titer. The sera of the other ten individuals are pending assay. Results of these assays should give some indication of whether IF levels can be correlated with time following immunization and/or with the immunization regimens employed.

Supernatant fluids from PHA-stimulated cultures initiated at various times from J.H.C., who was immunized with yellow fever vaccine, were simultaneously titrated as abc e. Results of interferon assays, as well as SI in parallel labeled cultures, are shown in Table 4. A sharp rise in IF production was noted approximately two weeks after immunization. (Not indicated in the table is the fact that supernates from nonstimulated cultures had no interferon-like activity.) A similarly increased interferon production over the preimmunization and early postimmunization levels persisted for at least three weeks. Endpoints were not reached for three of the test preparations, and assays will be repeated by the plaque reduction technic to determine the precise IF titer of these culture supernates.

It is to be noted that no correlation exists between the levels of PHA-stimulated lymphocyte transformation and IF induction. This observation is in disagreement with the statement of Wheelock and Toy (2) that "The degree of lymphocyte stimulation produced by either PHA or PPD as measured by the incorporation of uridine-^{3}H into RNA, correlated with the amount of interferon produced." On the other hand, Epstein et al. (3) report no significant correlation between the extent of PHA-stimulated blastogenesis and specific viral induction of IF in cells of sensitized individuals. Similarly, Pidot et al. (4) found no correlation of Newcastle disease virus-induced IF production and degree of PHA-induced transformation of cells from normal subjects. Furthermore, Wallen (5) showed that mouse spleen cells could be separated by albumin discontinuous gradient into a fraction which produced IF in response to non-specific mitogens (PHA and Concanavalin A) and which belonged to a different population of cells from those undergoing a DNA synthetic response to these mitogens.

Table 4. Titration of Interferon in Supernatant Fluids of PHA-stimulated Lymphocyte Cultures.

Cultures initiated	Stimulation index	IF titer
Day 0	34.2	160
Day 2	112.8	160
Day 9	14.7	160
Day 12	35.3	$\geq$ 1280
Day 19	47.1	$\geq$ 1280
Day 43	24.9	$\geq$ 1280

(Above individual received yellow fever vaccine)

Supernates from leukocyte cultures of one individual who received multiple vaccines have similarly been assayed for IF. Again, endpoints were not reached at dilutions of 1:640, the maximum tested. Reassay of these culture fluids, as well as all other collected, is pending.

MOUSE STUDIES. Inbred $C_{57}Bl/6J$ mice (obtained from Jackson Laboratories, Bar Harbor, Maine) were chosen as a laboratory model for our studies. This choice was predicated on the fact that the interferogenic response of mice apparently is quantitatively related to their genetic make up, and Kanady and Smith (6) showed the $C_{57}Bl$ strain to produce high titers of circulating IF in response to Newcastle disease virus (NDV).

NDV was passaged in embryonated eggs by inoculation into the chorioallantoic cavity. Harvested allantoic fluids were pooled, clarified by low speed centrifugation, and volumes suitable for subsequent use were stored at -40°C. This was found to be a good inducer of circulating interferon in mice when inoculated by the intraperitoneal route (a mode of inoculation technically easier than that by the intravenous route). Maximum titers were obtained 7 hours after infection.

PHA-P of a single lot in an amount adequate to complete our studies was obtained. After a vial was reconstituted, aliquots were stored at -40°C until use in order to retain activity. This mitogen was repeatedly titrated in normal mouse spleen cell cultures, using 0.1 ml of diluted mitogen/1 ml culture containing 2 X 10^6 cells and employing triplicate cultures in assay. Control cultures received diluent in place of mitogen. All cultures were incubated, pulsed and terminated as described for human peripheral leukocyte cultures. Reproducible stimulatory titers of 1:160 - 1:320 have been obtained. Results of one such titration are shown in Table 5. It is seen that high dosages of the mitogen were inhibitory, as has been reported by numerous investigators for cells of various animal species. Of interest is the fact that a 1:20 initial dilution of this lot of PHA-P - which effected an 86% reduction in isotope incorporation as compared with that in unstimulated cultures of mouse spleen cells - was highly stimulatory rather than toxic for human peripheral leukocyte cultures.

Table 5. Titration of PHA-P in Mouse Spleen Cell Cultures.

Initial PHA dilution	Average dpm,* 2×10^6 cells
1:20	496
1:40	1670
1:80	9723
1:160	19,950
1:320	20,357
1:640	16,549
1:1280	9792
Control cultures	3465

Assay system: 0.1 ml dilution of PHA plus 2×10^6 cells/ml.

* Average of triplicate cultures.

One of our objects was to determine the blastogenic and interferogenic action of not only PHA but also specific viral antigens in cultures of spleen cells from mice which had received various immunogens. To accomplish this aim, it was necessary to establish the optimal antigenic stimulus. For this purpose, mice were immunized with A/England/42/72 influenza virus by intramuscular injection of 0.1 ml of a concentrate containing 3600 hemagglutinating units/ml. On initial efforts to induce blastogenesis with this antigen, it was found to be highly toxic for cultures of spleen cells of the immunized mice. This cytotoxicity was eliminated by dialysis, and optimal blastogenesis as measured by thymidine uptake was obtained with a 1:512 dilution in an assay system consisting of 0.1 ml antigen and 2 X 10^6 cells in a total volume of 1 ml/culture. This stimulus is in marked contrast to that optimal for the A/Aichi virus preparation (12,800 hemagglutinating units/ml) in leukocyte cultures from our immunized human subjects (0.1 ml of a 1:16 dilution/10^6 cells).

Additional mice have been immunized with mumps virus skin test antigen. Determinations of specific blastogenesis in response to this antigen are currently in progress.

Another shipment of mice has been received. Our immediate plans are to immunize them with (1) multiple antigens (including mumps and influenza viruses), (2) single antigens, (3) placebo and (4) no immunogen (as a control for stress induced by handling). Animals from each group will be sacrificed sequentially for determination of (1) antibody response to the vaccine(s), (2) <u>in vivo</u> IF production in response to NDV, (3) <u>in vitro</u> response of spleen and lymph node cultures to specific and nonspecific mitogens, and capacity of spleen and lymph node cultures to produce IF upon stimulation with PHA, influenza and mumps viruses.

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