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Selective Increase in Lymphocyte Interferon Response to Vaccinia Antigen after Revaccination

(macrophage–lymphocyte interaction/mediator of cellular immunity)

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ABSTRACT **Viral antigen prepared by heat inactivation of vaccinia virus stimulated production of interferon in association with transformation of sensitized human lymphocytes *in vitro*. Involvement of a macrophage–lymphocyte interaction in production of interferon stimulated by viral antigen was found in which macrophages greatly augmented the amount of interferon produced by lymphocytes. Reimmunization with live vaccinia virus resulted in a selective increase in the ability of lymphocytes to produce interferon in the presence of viral antigen 4–7 weeks later without a corresponding increase in the degree of already significant lymphocyte transformation. There was no correlation between the extent of lymphocyte transformation and the amount of interferon produced. The augmented interferon response after reimmunization described in this study may be a significant component of the protective effect of immunization with vaccinia against disease occurring after exposure to smallpox.**

Epstein *et al.* demonstrated that a macrophage–lymphocyte interaction is involved in the mitogen- (1, 2) and antigen- (3, 4) stimulated production of interferon in cultures derived from human peripheral blood. Such interferon production occurred in association with the transformation of small lymphocytes to large blast-like cells observed in response to the nonspecific mitogen, phytohemagglutinin (PHA), and in the more specific immune reaction when sensitized lymphocytes transform in response to purified protein derivative (PPD). In both situations, lymphocytes were identified as the most likely cells responsible for the production of interferon, but the presence of macrophages greatly augmented the amount of interferon produced.

Numerous authors have reported the transformation of sensitized human lymphocytes in response to smallpox vaccine (5–11). With one exception, the smallpox vaccine used contained live vaccinia virus, and the possibility that replication of vaccinia in leukocytes contributed to the response could not be ruled out. Many other studies have demonstrated interferon production in cultured leukocytes stimulated by vaccinia virus (12, 13) as well as by other viruses (14, 15). Furthermore, cultures of leukocytes from animals immune to a given virus produced greater amounts of interferon when exposed to that virus than similar cultures from nonimmune animals (15). The present studies were therefore undertaken to answer the following questions:

(*a*) Can human lymphocytes transform and produce interferon on an immune specific basis in response to a nonreplicating vaccinia virus antigen?

(*b*) Is involvement of a macrophage–lymphocyte interaction necessary in the production of interferon as has been observed with mitogen- and nonviral-antigen-stimulated interferon production?

(*c*) Would reimmunization of donors with live vaccinia virus affect the degree of lymphocyte transformation and/or interferon production that occurred *in vitro* in response to the viral antigen?

It was hoped that such experiments would lead to greater understanding of both the cellular immune response to virus infections, and the mechanisms involved in viral immunizations.

Abbreviations: PHA, phytohemagglutinin; VH, heat-treated vaccinia antigen; PPD, purified protein derivative.

MATERIALS AND METHODS

Blood Donors. Healthy individuals whose leukocyte and differential counts were within normal limits, and who had no history of recent viral illness, served as blood donors. Eight of them were adults who had been immunized with vaccinia virus 2–30 years before the onset of the study and five of them were children who served as nonvaccinated negative controls. Vaccination had been contraindicated in the children because of a personal or sibling's history of asthma and/or atopic dermatitis. Blood samples from the adults were drawn before and 4–22 weeks after reimmunization.

Reimmunization of Adult Donors. By the multiple pressure technique, the adult donors were reimmunized with live vaccinia virus (Dryvax, Wyeth Laboratories). Vaccinations were performed as part of periodic routine physical examinations or in anticipation of foreign travel. The extent of erythema, induration, and vesicle formation at the site of vaccination were observed daily.

Preparation of Vaccinia Antigen (VH) and Vaccinia Antigen Control (VH Control). Vaccinia virus (obtained in the form of a vaccine lot from Cutter Laboratories) was passed repeatedly in HeLa-cell monolayers. Extracellular virus was collected at the time of maximum cytopathogenic effect and infectivity was determined by the extent of plaque formation on HeLa-cell monolayers. The virus pool with the highest titer, 4×10^5 plaque-forming units/ml, was then inactivated by heating at 56° for 3 hr, 1 hr in excess of the time necessary for complete inactivation. Complete inactivation was verified by inability of the inactivated virus to produce plaques on HeLa-cell monolayers. Such heat-inactivated preparations were used as vaccinia antigen; a dose of 0.1 ml per 2 ml of culture was used. The sterility of such preparations was con-

firmed by lack of bacterial growth in aerobic and anaerobic test cultures.

VH control was prepared in the same manner as VH except that uninfected HeLa-cell supernatants were used, passed repeatedly, and incubated at 56° for 3 hr. Likewise, A dose of 0.1 ml per 2 ml of culture was used. The cultures treated with VH control were used for demonstration of the degree of response to any possible HeLa-cell antigens that might also have been present in the VH preparations.

Preparation of Phytohemagglutinin (PHA). PHA-P (Difco) was prepared as described (1). The final concentration in culture was 34 μg of PHA-P per ml.

Preparation of Cultures. For each time point studied, two blood samples from each donor were needed. The first sample was used for preparation of pure macrophage cultures and the second sample, taken 1 week later, was used for preparation of pure lymphocyte cultures as described (1). The macrophage cultures (96–100% pure) resulted from the growth and differentiation of glass-adherent monocytes over a 7-day period, and the lymphocyte preparations (99.5–100% pure) were obtained by their passage through a sterile nylon fiber column. Erythrocytes that accompanied the lymphocytes in the column effluent were lysed by a 10-min exposure at 37° to a solution containing 9 ml of 0.83% NH_4Cl and 1 ml Tris buffer (pH 7.65). They were then removed by washing the lymphocyte pellet twice with McCoy's medium containing human AB serum. Thus, from each donor, it was possible to prepare pure cultures of lymphocytes, macrophages, or the two cell types together with VH, VH control, PHA, or no added stimulant at the initiation of the cultures. The final concentration of lymphocytes was always 0.9×10^6/ml and of macrophages 0.5 to 1.0×10^5/ml. Combined lymphocyte–macrophage cultures were always autochthonous.

Harvesting Procedures. The cultures, in Leighton tubes with coverslips, were maintained in a CO_2 incubator at 37°. Cultures containing VH or VH control were harvested at 5, 7, and 9 days, those with PHA were harvested at 3 or 4 days. [^{3}H]Thymidine (New England Nuclear Corp.; 2.1 Ci/mmol), was added 1 hr before termination of each culture. At the time of harvest, cultures were passed through a 10-place filter holder (Hoefer Scientific Instruments) with each filter port containing a Whatman Glass Filter-Paper disc (W. and R. Balston, Ltd.) supported by a disc of Whatman no. 1 filter paper, and the supernatant culture fluid was collected in sterile vials for interferon assay. For determination of the degree of incorporation of [^{3}H]thymidine into DNA as a measure of lymphocyte transformation, the cell pellets were then washed twice with cold phosphate buffered saline (pH 7.4), cold 5% trichloroacetic acid, and cold absolute methanol. The glass filter papers were then transferred and kept in scintillation vials containing 1 ml Nuclear-Chicago Solubilizer for several hours. 10 ml of scintillation fluid were then added to each vial, and the samples were counted as described (1).

Interferon Assay. A plaque reduction assay with fibroblasts from human neonatal foreskin and bovine vesicular stomatitis virus was used for interferon assay (1, 16). Interferon titer was defined as that dilution of sample which, in a 4-ml volume, resulted in 50% reduction of viral plaques. In most cases, duplicate samples were run for each type of culture. Control culture supernatants with medium or medium plus VH, VH control, or PHA were also tested. At least two, and most often three, dilutions of each sample were run along with the controls of the interferon assay, i.e., fibroblast monolayers with virus and no sample to be tested, and fibroblast monolayers with virus and dilutions of a known interferon standard.

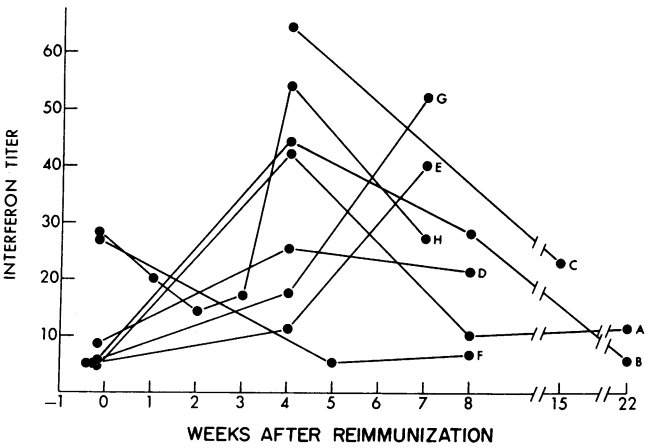

FIG. 1. Effect of reimmunization with live vaccinia virus on VH-stimulated interferon production in combined lymphocyte–macrophage cultures. For each donor at each time point combined cultures were examined at 5, 7, and 9 days after addition of VH, and the means of the values observed on the day of maximum reponse are plotted for each time interval. The letters *A–H* refer to individual donors.

RESULTS

Effect of reimmunization on interferon production

Fig. 1 depicts the maximum interferon titers in combined lymphocyte–macrophage cultures that were stimulated with VH at various time intervals before and after reimmunization. Before reimmunization, 5 of 7 donors (whose previous immunization was 2–30 years ago) had no detectable interferon titer. After reimmunization with live vaccinia virus, 7 of 8 donors had marked increases in interferon titers when studied 4–7 weeks later, followed by a decline of titer.

The data was subjected to statistical analysis by the Wilcoxon signed rank test. This test demonstrated that the increase in interferon titers observed after reimmunization was significant, with $P < 0.025$.

Effect of reimmunization on lymphocyte transformation

Fig. 2 depicts the maximum degree of [^{3}H]thymidine incorporation into DNA (as a measure of lymphocyte transformation) in combined lymphocyte–macrophage cultures that were stimulated with VH at various time intervals before and after reimmunization of the same donors depicted in Fig. 1. Before reimmunization, all donors manifested significant lymphocyte transformation in response to VH, with a 13- to 137-fold increase over that observed in cultures prepared with no stimulant present. After reimmunization with live vaccinia virus, significant amounts of transformed lymphocytes were again observed, but neither consistently higher nor lower than the amounts observed before reimmunization. For example, at 4 weeks three donors showed higher and three showed lower amounts of transformation than those observed before reimmunization. The degree of lympho-

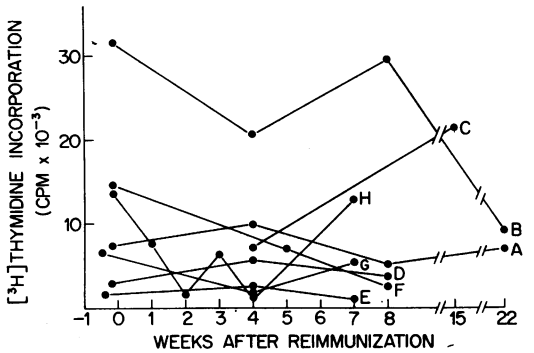

FIG. 2. Effect of reimmunization with live vaccinia virus on VH-stimulated lymphocyte transformation (as determined by the incorporation of [³H]thymidine incorporation into DNA) in combined lymphocyte–macrophage cultures. For each donor at each time point combined cultures were examined at 5, 7, and 9 days after the addition of VH. For each time point, the values plotted are the means of values observed on the day of maximum response less those observed in combined cultures prepared with no stimulant. The letters *A–H* refer to individual donors.

cyte transformation both before and after reimmunization was always significantly higher than that observed in cultures prepared with no stimulant. Similarly, the response seen in VH-treated combined cultures was at all times significantly higher (2- to 26-fold) than in combined cultures treated with VH control. No interferon was noted in VH control-treated cultures.

Relationship between VH-stimulated interferon production and lymphocyte transformation

Two representative time-course studies performed 4 weeks after reimmunization are presented in Fig. 3. For each donor combined lymphocyte–macrophage cultures were examined 5, 7, and 9 days after the addition of VH. Both experiments demonstrate the dissociation in time of peak [³H]thymidine incorporation and maximum interferon production. Maximum interferon production always occurred at least 2 days after peak [³H]thymidine incorporation. A similar dissociation in

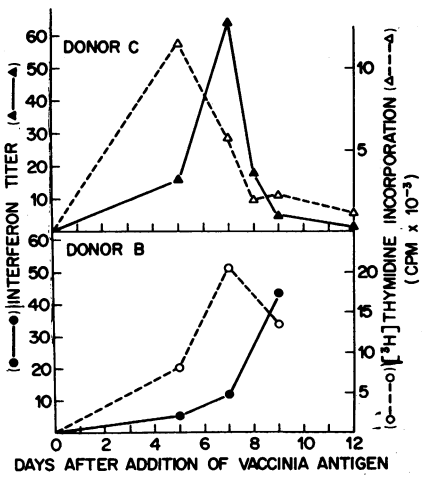

FIG. 3. Time-course studies of VH-stimulated interferon production and incorporation of [³H]thymidine in combined lymphocyte–macrophage cultures 4 weeks after reimmunization with live vaccinia virus. The values plotted are the means of duplicate samples for each time point.

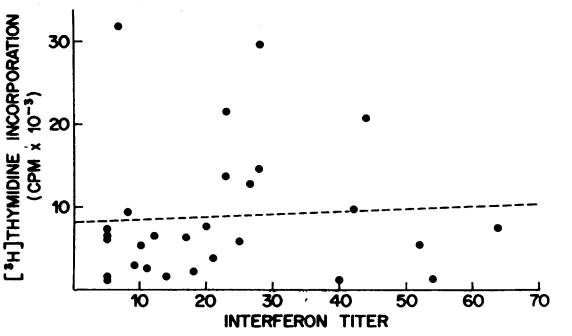

FIG. 4. Relationship between maximum interferon titer and peak [³H]thymidine incorporation. The *line* shown is computed by linear regression analysis.

time between these two parameters of the *in vitro* cellular response to an antigen was reported previously with PPD in combined cultures from sensitized individuals (3, 4). In that situation also, maximum interferon production followed peak incorporation of [³H]thymidine.

Fig. 4 depicts the results when such maximum values for VH-stimulated incorporation of [³H]thymidine and interferon production for all donors at every time interval before and after reimmunization are studied. Linear regression analysis showed no significant correlation between the extent of these two phenomena.

Relationship between the extent of formation of skin lesions and maximum lymphocyte transformation or interferon production

No correlation between the extent of erythema, induration, or vesicle formation at the site of vaccination was found with either the extent of maximum lymphocyte transformation or with maximum interferon production.

Effect of macrophages on VH-stimulated lymphocyte transformation and interferon production

A comparison of the extent of [³H]thymidine incorporation into DNA in VH-stimulated cultures prepared with lymphocytes alone and those prepared with both lymphocytes and macrophages is demonstrated in Fig. 5. With one exception (donor B at 22 weeks), the addition of macrophages to cultures of lymphocytes always greatly augmented the degree of lymphocyte transformation in the presence of VH. Similarly, the presence of macrophages always augmented the amount of interferon produced over that observed in cultures of VH-stimulated lymphocytes alone. With the one same exception mentioned previously, the titer of interferon in VH-stimulated lymphocyte cultures was always <10, whereas in the combined cultures values as high as 64 were observed. When lymphocytes were subjected to repeated freeze-thawing before combination with macrophages and either PHA or VH, no significant transformation or interferon production was observed. However, when macrophages were subjected to repeated freeze-thawing before combination with lymphocytes and either PHA or VH, a small interferon response was noted.

Studies with nonvaccinated donors

The data in Table 1 indicate that, whereas all but one donor (YW) were capable of responding to the nonspecific mitogen PHA with significant lymphocyte transformation (as mea-

sured by the incorporation of [^{3}H]thymidine into DNA) and with interferon production, none of the donors showed a significant response to VH. The lack of responsiveness of YW to PHA could possibly be attributed to her therapy or to her asthma and atopic dermatitis.

Physical and chemical characterization of the interferon produced in combined lymphocyte–macrophage cultures with VH

As was demonstrated with PHA- and PPD-stimulated interferon (1–4), VH-stimulated interferon was destroyed by the action of trypsin. Its activity was unaffected by ultracentrifugation at 100,000 × *g*, but was depressed by 50% by heating at 56° for 1 hr. Considerable activity was lost after exposure of the interferon to pH 2 for 6 hr. Species specificity was demonstrated by the inability of the VH-stimulated interferon to confer protection on feline fibroblasts, when challenged with vesicular stomatitis virus. However, barely detectable levels of protection were afforded mouse L cells. Exposure of the confluent monolayers of neonatal foreskin fibroblasts to the culture supernatants that contained interferon for only 2 hr instead of the customary 18–24 hr was not sufficient for the interferon to exert its protective effect.

DISCUSSION

The present studies demonstrate that it is possible to prepare a viral antigen from vaccinia virus that will result in transformation of sensitized lymphocytes and in an associated production of interferon *in vitro*. Although the precise antigenic composition of the VH used in these studies is not known, three lines of evidence support the conclusion that the events observed *in vitro* were a direct consequence of a specific immunologic response to VH. First, no significant lymphocyte transformation or interferon production was noted in cultures prepared from nonvaccinated donors. However, that their lymphocytes could transform and make interferon if given a proper stimulus was demonstrated by their positive response to the nonspecific mitogen, PHA. Second, no significant lymphocyte transformation and interferon production were observed in VH control-treated cultures from vaccinated donors. Thus, it is not likely that positive responses noted with VH in cultures derived from these same donors

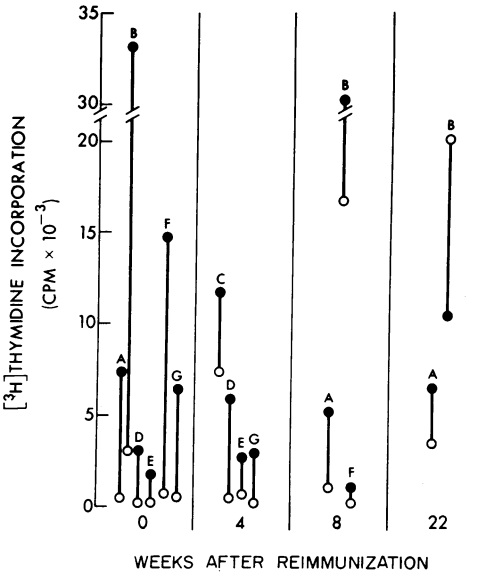

FIG. 5. Effect of macrophages on incorporation of [^{3}H]thymidine into DNA in response to VH. (●), VH-stimulated cultures that contained macrophages and lymphocytes; (○), VH-stimulated cultures of lymphocytes.

TABLE 1. *Effect of VH and PHA on lymphocyte transformation and interferon production in cultures derived from nonvaccinated donors*

	Donors									
	J.H.		S.M.		J.M.*		M.W.		Y.W.	
Type of culture	Interferon titer	[^{3}H]Thymidine incorporation (cpm)	Interferon titer	[^{3}H]Thymidine incorporation (cpm)	Interferon titer	[^{3}H]Thymidine incorporation (cpm)	Interferon titer	[^{3}H]Thymidine incorporation (cpm)	Interferon titer	[^{3}H]Thymidine incorporation (cpm)
M + L†	<10	96	<10	183	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
M + L + PHA‡	13	37,000	35	20,800	20	17,500	N.D.	N.D.	N.D.	N.D.
L	<10	73	<10	328	<10	88	<10	169	<10	3460
L + PHA	50	22,700	10	11,100	<10	12,700	53	29,200	<10	2190
M + L	<10	403	N.D.*	N.D.	N.D.	N.D.	<10	381	<10	271
M + L + VH§	<10	721	<10	320	<10	876	<10	885	<10	179
L	<10	489	<10	447	<10	407	<10	83	<10	533
L + VH	<10	721	<10	71	<10	221	<10	277	<10	286

* Because of young age of some donors and limited amount of blood available, it was not possible to prepare all combinations of cultures. N.D., not done.

† M, cultures containing macrophages; L, cultures containing lymphocytes.

‡ All cultures containing PHA and their controls were harvested at 3 or 4 days.

§ All cultures containing VH and their controls were harvested at 7 days.

J.H. Age, 7.75 years. History of eczema, asthma at age 1–2. Now asymptomatic and off therapy.

S.M. Age, 7.75 years. History of eczema, chronic bronchitis. Now asymptomatic and off therapy.

J.M. Age, 3.5 years. Normal sibling of S.M.

M.W. Age, 11 years. Normal sibling of Y.W.

Y.W. Age, 9.75 years. History of eczema, asthma, chronic cough. On therapy with oral ephedrine.

could be attributed to their reaction to HeLa-cell antigens. Third, a definite increase in *in vitro* interferon production was noted in response to reimmunization with live vaccinia virus. 4–7 weeks after reimmunization, 7 of 8 donors showed a significant increase in the amount of interferon stimulated *in vitro* by VH.

These studies also demonstrate the involvement of a macrophage–lymphocyte interaction in *in vitro* production of viral antigen-stimulated interferon that is similar to that observed with PHA- or PPD-stimulated interferon (1–4). The presence of macrophages in VH-stimulated, combined cultures greatly augmented the degree of transformation and interferon production over that observed with just lymphocytes alone. As had been observed with PHA and PPD (1–4), no interferon production was ever noted in VH-stimulated cultures containing macrophages alone. This, plus the fact that repeated freeze-thawing of lymphocytes before combination with macrophages and PHA or VH completely prevents lymphocyte transformation and subsequent interferon production, strongly suggests that lymphocytes are the cell-types responsible for *in vitro* production of interferon in response to a viral antigen.

From the present data, it is not possible to determine the exact mechanism of the increased interferon response observed after reimmunization. Yet, it seems reasonable to assume that it occurred either because of an increase in the number or productive capacity of the lymphocytes that produce interferon. Furthermore, they might be a separate population from, or a subpopulation of, these lymphocytes that respond to viral antigen with lymphocyte transformation and proliferation. This hypothesis is supported by the observations, in the present study, that reimmunization with live virus did not result in an increase in the already significant levels of lymphocyte transformation stimulated by viral antigen, observed before reimmunization, but did result in an increase in the ability of sensitized lymphocytes to respond to viral antigen with the increased production of interferon.

The latter observation may well provide a new explanation for the protection against smallpox that is afforded by revaccination. The importance of cell-mediated immunity in defense against pox viruses has been suggested in man (17, 18) and animals (19–22). Interferon seems to function as a mediator of cellular immunity (23, 3, 4) and has been detected at the dermal site of vaccination (24, 25). Also, enhanced local interferon production by immunized mice has been observed after intracranial challenge with vaccinia (26). That such local interferon may play a very important role in recovery or resistance to reinfection has been documented by studies of *Varicella zoster* (another enveloped DNA virus affecting the skin) in patients with impaired cell-mediated immunity (27). It was shown, in such patients, that a delay in appearance of interferon at the local site was associated with severe progressive infections and that the appearance of high local titers of interferon signaled the onset of recovery.

Thus, the phenomenon reported herein, that reimmunization with live virus results in increased interferon production by sensitized lymphocytes, may be of critical importance in local areas with high concentrations of viral antigens, and thereby contribute to the protective effect of smallpox vaccination. Local interferon production as a component of cellular immunity may also function together with humoral immunity in recovery from viral disease.

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