

IMMUNE INTERFERON PRODUCED TO HIGH LEVELS BY ANTIGENIC STIMULATION OF HUMAN LYMPHOCYTES WITH INFLUENZA VIRUS

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We detected two types of lymphocyte-mediated cytotoxicity following antigenic stimulation *in vitro* of lymphocytes from volunteers who had recently received a live or inactivated influenza vaccine. In addition to detecting an increase in the influenza virus-specific HLA-restricted cytotoxic T cell response, we observed a lesser degree of cytotoxicity of target cells that was neither virus specific nor HLA restricted.¹ It was known that influenza infection *in vivo* (1, 2) and *in vitro* induced interferon (3, 4), so we speculated that the nonspecific cytotoxicity was due to augmented natural killer cell activity of the cultured lymphocytes by interferon production in the culture.

The titers of interferon in the supernatant fluids of the cultures were >10,000 U/ml. This led us to measure the production of interferon by influenza virus stimulation of the lymphocytes of individuals who had not been vaccinated recently. In addition we analyzed the kinetics of interferon induction, the nature of the influenza antigen required, and the type of interferon produced.

Materials and Methods

Virus Preparations. Virus strains were grown in the allantoic sac of embryonated hens' eggs and were kindly provided by Dr. G. C. Schild, National Institute for Biological Standards and Control (NIBSC), London, England. The strains used were A/PR/8/34 (HON1), A/Munich/1/79 (H1N1), A/Port Chalmers/1/73 (H3N2), B/Hong Kong/5/72, A/Chick Germany/N/49 (H10N7), A/Turkey Wisconsin/1/66 (H9N2), A/Equine/Prague/56 (H7N7), and A/Equine 2/Miami/63 (H3N8). Whole virus vaccine concentrates containing 330 µg, respectively, of A/Port Chalmers/1/73 or B/Hong Kong/5/72, hemagglutinin (HA) and a surface antigen vaccine that contained 194 µg of A/England/321/77 (H3N2) hemagglutinin were provided by Dr. John Wood, NIBSC. Purified HA, obtained by disruption with Triton X-100 followed by fractionation on 20–50% (wt/vol) sucrose gradients, and prepared as described (5) from A/Singapore/57 (H2N2), A/England/1/72 (H3N2), MRC-11 (H3N2), and a recombinant with Equine 1 HA and USSR neuraminidase designated H Eq1N1 (H7N1) virus strains, were kindly given to us by Dr. J. Oxford (NIBSC).

Stimulation of Lymphocytes. Approximately 50 ml of peripheral blood containing 50 U of preservative-free heparin was diluted with a half volume of medium (RPMI 1640, Gibco Laboratories, Grand Island Biological Co., Grand Island, N.Y.) and layered onto Ficoll-Paque (Pharmacia, Uppsala, Sweden). These preparations were centrifuged at 1,400 rpm for 30 min, and the layered buffy coats were removed and washed with the same medium three times. The

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cells were then counted and resuspended in the same medium containing 10% fetal calf serum. Some of these lymphocytes were used in experiments that analyzed the HLA-restricted influenza-specific cytotoxic T cell response to vaccination.¹ The lymphocytes used in the present studies were resuspended into a concentration of 1.0×10^6 cells/ml of medium with 10% fetal calf serum. 10% of the cells were exposed to 1 ml of either live virus (~1,000 hemagglutinating units of allantoic fluid-prepared virus), inactivated vaccine, or preparations of purified HA diluted with phosphate-buffered saline (pH 7.2) to contain 30 μ g of HA/1.0 ml added to the stimulator cells, undiluted allantoic fluid, or medium alone. The aliquots of 10% of the cells were incubated with these various preparations for 90 min at 37°C in 5% CO₂. These stimulator cells were then washed twice in medium with 10% fetal calf serum, and added to the remaining 90% of the cells (responders). The mixed cells were cultured at 37°C in 5% CO₂ for up to 7 d. The details of these methods have been described earlier, using both murine lymphocytes (6–8) and human lymphocytes (9, 10) exposed to influenza virus as stimulators of influenza-specific cytotoxic T cell activity.

Interferon Assay. Interferon was assayed by a cytopathic effect reduction assay (11). Threefold serial dilutions of interferon samples were incubated with Hep2C cells for 20 h at 37°C and then challenged with encephalomyocarditis virus. An internal interferon standard was included in each set of assays, and titers read after a further 48 h at 37°C. The international reference preparation of human leukocyte alpha interferon (69/19) reproducibly titered at 3,000 U/ml in this assay; i.e., 1 laboratory unit was equivalent to 1.67 IU/ml with respect to the 69/19 human leukocyte interferon standard in the absence of a recognized international reference preparation for human gamma interferon.

Neutralization of Interferon. Neutralization of antiviral activity was carried out by using specific antisera to human alpha and beta interferons. Calf and sheep antihuman interferon [α (Ly)] antisera were obtained by immunizing animals with partially purified human lymphoblastoid interferon [HuIFN α (Ly)] obtained from Wellcome Research, Beckenham, Kent, Eng. Rabbit antihuman FS4 fibroblast beta interferon was a gift from Dr. J. Vilcek, Dept. of Microbiology, New York University School of Medicine, New York. Diluted interferon samples containing 10 U/ml were incubated for 2 h at room temperature with serial dilutions of antiserum, and then the residual interferon was titrated in the CPER assay. The neutralizing titer of the antiserum is expressed as the dilution of antiserum required to totally ablate the antiviral activity of an interferon preparation containing 10 U/ml.

Results

The interferon titers summarized in Table I were obtained on supernatant fluids of lymphocyte cultures, prepared from the lymphocytes of volunteers who had been given influenza vaccine 14 and 180 d earlier. There were 36 individuals who had received either a live attenuated vaccine, or one of two types of inactivated vaccine, whole virus, or surface antigen. Results indicate that the lymphocytes of the volunteers, after *in vitro* exposure to stimulator lymphocytes treated with live virus (A/Munich/1/79 [H1N1], antigenically similar to the virus strains in the vaccines), produced interferon, and lymphocytes not exposed to the stimulator lymphocytes did not. Lymphocytes from individuals given any of the three vaccines produced high amounts of interferon after exposure to stimulator lymphocytes when the culture fluids were tested 7 d later. The mean interferon responses were above 10,000 U/ml for each vaccine group at 14 d after vaccination. This decreased somewhat by 180 d after vaccination, when the mean responses of the volunteers lymphocytes were ~4,000 U/ml for the three vaccine groups.

We next examined the production of interferon by influenza virus stimulation of lymphocytes of normal adult blood donors, who had not received influenza vaccine. Table II summarizes the titers of interferon measured in these lymphocyte cultures. It is clear that cultured lymphocytes exposed to virus-treated stimulator cells produced

TABLE I
Interferon Production* by the Lymphocytes of Vaccinated Volunteers following In Vitro Stimulation with A/Munich/1/79 (H1N1) Virus

Volun- teer	Live vaccine		Intact whole virus			Surface antigen		
	14	180‡	Volun- teer	14	180	Volun- teer	14	180
12	4.3	2.7	13	3.8	2.6	14	4.5	3.4
15	4.0	2.6	16	4.3	2.7	17	4.0	2.2
21	4.0	3.2	19	4.0	3.4	20	4.0	3.0
24	4.5	2.9	22	4.0	3.3	23	4.5	2.4
27	4.5	2.2	25	4.0	—	26	4.5	3.4
30	4.5	4.1	28	4.0	3.3	29	2.5	2.8
33	4.0	2.9	34	4.5	3.7	32	3.5	2.4
36	4.0	—	37	3.5	2.5	35	4.5	1.2
39	4.5	—	43	4.5	3.2	38	4.5	3.6
42	4.5	4.2	46	4.0	3.3	41	4.5	—
45	4.0	2.8	49	4.0	4.7	50	4.5	4.4
51	4.5	2.8	52	4.0	1.4	54	4.0	—
Mean	4.34	3.5		4.13	3.78		4.34	3.6

* Interferon values are expressed as log₁₀ IU/ml.

‡ 14 and 180 designate the interferon produced by lymphocyte cultures established 14 and 180 d after vaccine was administered.

TABLE II
Interferon Production* by Lymphocytes of Platelet Donors by In Vitro Stimulation with Influenza Viruses

Donor	Virus added to stimulator cells				
	None	A/PC/1/73 (H3N2)	A/PR/8/34 (H0N1)	A/Munich/ 1/79 (H1N1)	B/Hong Kong/5/72
56	0	1.7	1.4	—‡	1.7
57	0	1.7	1.6	—	1.7
58	0	3.0	2.7	—	2.7
59	0	2.6	3.1	—	3.0
60	0	3.2	3.2	—	3.2
61	0	2.7	2.7	—	2.7
62	0	3.4	3.5	—	3.8
63	0	3.8	3.7	—	3.7
64	0	2.7	2.5	2.0	2.7
65	0	3.0	2.9	2.5	2.9
66§	0	2.5	2.0	—	2.0
67§	0	2.7	2.7	—	3.0
70§	0	2.5	2.5	—	3.0
75	0	2.2	—	—	2.7
76	0	2.5	—	—	3.5
61R	0	2.5	2.8	—	—
Mean		3.0	3.03	2.32	3.17

* Interferon values are expressed as log₁₀ IU/ml.

‡ —, not performed.

§ Indicates interferon sample was obtained 3 or 5 d after stimulation instead of 7 d.

TABLE III
Interferon Production by Human Lymphocytes Stimulated by Human Influenza Viruses, Vaccines, Purified HA, and by Nonhuman Influenza Viruses*

Virus added to stimulator cells	Donors					
	62	63	68	69	75	76
Human viruses						
A/PR/8/34 (H0N1)	3.5	3.6	2.7	3.0	—	—
A/PC/1/73 (H3N2)	3.3	3.5	2.5	2.7	2.2	2.5
B/Hong Kong/5/72	3.5	3.5	3.7	2.7	2.7	3.5
Nonhuman viruses						
A/Eq/1/56	3.0	3.2	—	—	—	—
A/Eq/2/63	3.0	3.2	2.7	2.4	—	—
A/Turkey/N/66	3.2	3.4	2.5	2.7	—	—
A/Chick/N/49	3.2	3.6	2.9	2.7	—	—
Inactivated vaccine						
A/Port Chalmers/1/73	—	—	—	—	1.0	1.7
B/Hong Kong/5/72	—	—	—	—	1.5	2.0
A/England/1/72	—	—	—	—	0	0
surface antigen vaccine						
Purified HA						
A/Sing/57 (H2)	0	0	—	—	—	—
A/Eng/1/72 (H3)	—	—	0	0	—	—
MRC-11 (H3)	—	—	0	0	0	0
H/Eq1N1 (Eq1)	—	—	0	0	—	—
Allantoic fluid	—	—	0	0	—	—
Controls	0	0	0	0	0	0

* Interferon values are expressed in \log_{10} IU/ml and were measured 7 d after stimulation.

interferon, and that such stimulation was necessary for production. Several influenza virus strains were used to infect stimulator lymphocytes, and all induced interferon. The lymphocytes of some donors produced higher levels of interferon to all of the virus strains, whereas others produced somewhat lower levels. The level of production of interferon was similar for any one lymphocyte donor after stimulation by any of the virus strains. Repeat testing of the lymphocytes from one donor 3 mo later gave similar interferon titers (61 and 61R).

The results in Table II indicated that stimulator cells exposed to several influenza A viruses of the H0N1, H1N1, and H3N2 subtypes, as well as influenza B, stimulated interferon production. The results of exposure of responder lymphocytes in vitro to stimulator lymphocytes treated with live infectious human virus, inactivated whole or surface antigen vaccine, purified HA preparations, and live nonhuman viruses, are contained in Table III. Lymphocytes cultured without virus-treated stimulator cells, following treatment with allantoic fluid-treated stimulator cells, or following treatment with medium-treated stimulator cells, did not produce interferon. All lymphocyte cultures with live, human or nonhuman, virus-treated stimulator lymphocytes produced interferon. Two inactivated whole virus vaccines induced some, but lower, levels of interferon, and no interferon was detected in the cultures containing stimulator cells treated with an inactivated surface antigen vaccine. No interferon was produced when stimulator lymphocytes were exposed to preparations of purified HA prepared from an H2N2, H3N2, or Heq1N1 virus strain. These results indicate

TABLE IV
Time-Course of Interferon Production by Lymphocytes Stimulated by Influenza*

Donor	Virus added to stimulator cells	Time after stimulation			
		Day 1	Day 4	Day 7	
64	A/Port Chalmers (H3N2)	2.7	2.7	2.7	
	A/Munich (H1N1)	2.2	2.2	1.95	
	A/PR/8 (H0N1)	2.7	2.7	2.45	
	B/HK/5/72	2.95	2.9	2.65	
66	A/Port Chalmers (H3N2)	Day 1	Day 5		
		1.7	2.45		
		1.2	1.95		
	B/HK/5/72	1.2	1.95		
70	A/Port Chalmers (H3N2)	1.5 h	4 h	24 h	72 h
		0	0	1.9 (1.4)‡	2.5 (0)§
		0	0	1.9 (1.4)	2.5 (0)
		0	0	2.1 (1.65)	3.0 (0)
		0	0	2.1 (1.65)	3.0 (0)

* Interferon values are expressed as log₁₀ IU/ml.

‡ Units of interferon measured after 24 h in culture with a change in medium at 4 h are indicated in parentheses.

§ Units of interferon measured after 72 h in culture with a change in medium at 4 h and 24 h are indicated in parentheses.

TABLE V
Characteristics of Interferons Produced by Influenza-stimulated Lymphocyte Cultures

Donor	Stimulator viruses	Untreated	Interferon level pH 2 treatment*	Percent pH 2 labile
26	A/Munich/1/79 (H1N1)	4.2	3.2	90
63	A/PR/8 (H0N1)	3.2	2.5	80
	A/Eq1/56 (Heq1Neq1)	2.9	2.5	60
	A/Chick/N/49	3.5	2.7	84
	A/Turkey/N/46	3.1	2.5	75

* Interferon values are expressed as log₁₀ IU/ml.

that the stimulator cells must be exposed to intact virus in order for interferon to be stimulated in the cultures. Inactivated vaccines that contained intact virus particles also induced interferon, but to a lesser degree; surface antigen vaccine and purified HA preparations did not. It was interesting to observe that several nonhuman viruses also induced interferon to titers similar to those produced by the human viruses.

The production of interferon occurred within the first few days of culture and was stable or fell slightly by day 7, as shown in Table IV. Changing the medium after 4 h of culture reduced the yield, and changing medium at 4 and 24 h resulted in the absence of interferon in the cultures when tested at 72 h (Table IV).

The results shown in Tables III and IV suggested that interferon induction resulted from exposure of responder lymphocytes to stimulator cells treated with intact viruses. These stimulator cells induced interferon in the culture, but production of interferon

TABLE VI
*Effect of Treatment of Influenza-stimulated Lymphocyte Culture Fluids with
 Antiserum to Human Lymphoblastoid Interferon*

Donor	Virus stimulator	Antibody level*	
		Untreated	pH 2 treatment
63	A/PR/8 (H0N1)	3,000	1,000,000
	A/Eq/1/56 (Heq1Neq1)	100,000	1,000,000
	A/Chick/N/49	10,000	300,000
16	A/Munich/1/79 (H1N1)	<100	300,000
26‡	A/Munich/1/79 (H1N1)	<100	300,000

* Dilution of sheep antiinterferon [α (Ly)] which has a neutralizing titer of 300,000–1,000,000 against 10 IU human lymphoblastoid interferon, at which it neutralized 10 U of antiviral activity in the supernatant fluid. This antiserum was used in all assays except in the case of donor 16, when a similar calf antiserum to α (Ly) interferon was used.

‡ In this case interferon was passed through an NK2 monoclonal antibody to alpha interferon column (Secher and Burke, 13). The untreated sample is representative of the flow-through or unbound fraction, and the pH 2-treated sample is that fraction eluted with pH 2 buffer from the immunoabsorbent column.

appeared to be mediated by a factor released into the medium, the removal of which was associated with a decrease in interferon production.

Studies were performed to analyze the type of interferon induced. The results presented in Table V indicate that most of the interferon produced was labile at pH 2. Supernatant fluid from a culture of lymphocytes established 14 d after vaccination had a 90% drop in interferon titer after pH 2 treatment. Decreases were also produced in the interferon level after pH 2 treatment of the culture fluids of lymphocytes exposed to virus-treated stimulator cells of the donors not vaccinated (donor 63). These observations suggested that gamma (immune) interferon, which is known to be acid labile, was being produced in the lymphocyte cultures after exposure to virus-treated stimulator cells having influenza antigens on their membranes. Although some of the interferon remained after pH 2 treatment and may have been induced by virus infection rather than immune recognition, no infectious virus was detected in the interferon containing supernatant fluids when they were tested on Madin-Darby canine kidney cells, which are very sensitive for detecting live influenza viruses (12) (data not shown).

Treatment of several of the interferon-containing supernatant fluids with antisera to alpha interferons neutralized the pH 2 stable interferon (Table IV). The antisera did not decrease the activity of the interferon detected in untreated supernates of lymphocyte cultures obtained from recently vaccinated individuals (donors 16 and 26), but had some activity against the interferon produced in cultures of individuals who were not vaccinated. Antiserum to beta interferon had no effect on the interferon produced by lymphocyte cultures of either vaccinated or unvaccinated donors (data not shown). The data shown in Tables V and VI suggested that lymphocyte cultures exposed to influenza virus-treated stimulator cells contained mixtures of alpha and gamma interferons, with a larger component of gamma interferon, especially in the cultures of lymphocytes from the recently vaccinated volunteers.

In an attempt to further characterize the mixture of interferons, the supernate from donor 26 was passed through an NK2 monoclonal antibody to alpha interferon-

Sephadex 4B immunoabsorbent column (13). Only 5% of the interferon was bound to the column and this was eluted with a pH 2 buffer. The flow-through solution contained >90% of the interferon activity added to the column. This interferon remained pH 2 labile and was not neutralized by antiserum to alpha interferon (Table VI).

Partial purification of interferon in the pooled supernate of lymphocyte cultures from recently vaccinated volunteers by control pore glass and concanavalin A-Sepharose 4B chromatography (14) revealed two components. The first did not bind to concanavalin A-Sepharose, and the second was eluted with 0.1 M α -methyl-D-mannoside. This second component, comprising ~50% of the input interferon activity, had elution characteristics identical to gamma interferon from phytohemagglutinin-stimulated human lymphocytes purified in the same way.

Discussion

Our results indicated that high levels of immune interferon were produced by exposure of human lymphocytes in culture to stimulator cells that had been treated with influenza virus. The lymphocytes of individuals who had recently received influenza vaccine produced the highest levels of interferon, and their lymphocytes still produced high levels when tested 6 mo after vaccination. At 6 mo the level of interferon produced was still considerably higher than that detected in cultures of lymphocytes obtained from donors who had not received the vaccine. These data suggest that human lymphocytes with memory for influenza virus antigen(s) expand following exposure in vivo by vaccination. When subsequently exposed to influenza-treated stimulator cells in vitro, these memory cells further expand and produce large amounts of immune interferon. In the absence of recent antigenic stimulation in vivo, human lymphocyte cultures produce immune interferon following exposure to influenza virus-treated stimulator cells, but at a lower level. The interferons produced in the cultures were a mixture, but a high concentration of immune interferon was produced, especially in the cultures of lymphocytes from recently vaccinated individuals.

The responding lymphocytes apparently recognized influenza antigens on the stimulator cells. The nature of the antigen recognized has not been defined. It must, however, be shared by human and nonhuman influenza A viruses, because stimulator cells treated with either resulted in interferon production by the lymphocytes. The antigen was most efficient at inducing interferon when a live virus was used. Induction of interferon appeared to require exposure of stimulator cells to an intact virus, as purified HA and a surface antigen vaccine preparation containing HA and neuraminidase did not induce interferon. The need for intact virus suggests that the structure containing the stimulating antigen is important for proper presentation and/or processing of antigen to occur. These results indicated that isolated HA was not satisfactory for treating the stimulator cells for inducement of interferon, but nevertheless, the HA on the surface of the intact virus may be the antigen that is recognized. If HA antigen is responsible for inducing immune interferon production it must be by a cross-reactive determinant of the HA present on both human and nonhuman influenza viruses. It is also possible that a shared determinant of some other antigen, neuraminidase, nucleoprotein, or matrix was recognized by the responding lymphocytes and resulted in the production of interferon. Recent studies have

indicated that monoclonal antibodies to HA and neuraminidase bind to the murine myeloma cells P815 when they are infected with influenza viruses, and to a lesser degree antibody to nucleoprotein. Antibody to matrix did not bind (15). These types of experiments performed on human lymphocytes following infection with influenza virus might help define the nature of the antigen recognized by the responding memory lymphocytes.

The levels of gamma interferon produced in these experiments were much higher than has been previously reported. Addition of influenza virus directly to cultured human peripheral blood lymphocytes, without pretreating stimulator cells, resulted in the production of a few hundred units of pH 2-stable interferon, neutralized by an antiserum to alpha interferon, and appearing to be mainly produced by nonadherent cells (16). Other reports have also described the production of moderate amounts of classical virus-induced alpha interferon stable at pH 2 following virus stimulation of cultured lymphocytes (17–20). The results of the experiments reported herein contrast with previously published reports because much higher levels of pH 2-labile gamma interferon were detected following exposure of human lymphocytes to virus-treated stimulator cells in the present studies. We used this method for presenting virus antigen to memory T cells because it is a very effective way to stimulate the H2- or HLA-restricted influenza virus-specific cytotoxic T lymphocytes (CTL) response. This technique was used for measuring HLA-restricted CTL responses in the volunteers who had received the vaccines. This method has not been previously reported to result in high levels of gamma interferon production. Apparently, the recognition of influenza antigen on the stimulator cells by the responding lymphocytes resulted in increased immune interferon production as well as in stimulating the HLA-restricted virus-specific cytotoxic T cell response.¹ There may be an interrelationship between the induction of gamma interferon and the cytotoxic T cell response involving interleukin 2, as suggested by Farrer et al. (21) who reported that interleukin 2 appeared necessary for production of gamma interferon, and cytotoxic T cell responses in mixed leukocyte cultures.

Other methods of producing gamma interferon have generally used nonspecific mitogens, rather than antigens, as inducers. In general, the levels of gamma interferon that have been induced by stimulation with mitogens such as concanavalin A, phytohemagglutinin, and staphylococcal enterotoxin A have been ~ 100 – 800 U interferon/ 10^6 cells (22, 23). Mixed lymphocyte cultures have been reported to produce ~ 10 U/ 10^6 cells (24). These levels are much lower than the titers of immune interferon produced in the present studies, i.e., up to $50,000$ U/ 10^6 cells from most of the recently vaccinated individuals, and $\sim 4,000$ U/ 10^6 cells 6 mo after vaccination. The lymphocyte cultures of normal adult blood donors produced $\sim 1,000$ U/ 10^6 cells.

The explanation for the observation of such high levels of immune interferon production is not clear. It is possible that humans, as a result of repeated infection with influenza viruses, have been stimulated numerous times with the cross-reactive antigen recognized by memory T cells, resulting in these levels of immune interferon production. The further increase in immune interferon production obtained by using the lymphocytes of recently vaccinated individuals would support this hypothesis. It may also be speculated that the cross-reactive determinant recognized by and resulting in immune interferon production is the same determinant recognized by one population of cytotoxic T lymphocytes. This population, unlike the subtype-specific

cytotoxic antibody (25) and cytotoxic T cells (26, 27), kills target cells across influenza virus subtypes (28–30).

The ability to stimulate lymphocytes *in vitro* following recent *in vivo* stimulation is a method of antigenic stimulation of immune interferon that may be applicable to many antigens. The *in vitro* infection of stimulator cells appears, however, to produce the best yields of immune interferon as a response to influenza antigens. The levels of immune interferon produced by this method far exceed the levels of both alpha and gamma interferon previously reported following infection of lymphocyte cultures by viruses, including influenza (16–18), and by mitogens (21–23).

Summary

Influenza virus stimulation of human lymphocytes induced high levels of immune interferon in lymphocyte cultures. The lymphocytes of normal adults produced $\sim 1,000$ U/ 10^6 cells, which was in large part gamma interferon. The lymphocytes of individuals recently vaccinated yielded very high levels (10–50,000 U/ 10^6 cells) of interferon. The interferon was pH 2 labile, and was not neutralized by antisera to alpha or beta interferon. It did not bind to a monoclonal antibody to alpha interferon, and after partial purification it had characteristics identical to human gamma interferon induced by phytohemagglutinin. The highest yields were produced by treatment of stimulator cells with live virus. Stimulation by whole inactivated virus resulted in lower levels of interferon, and purified hemagglutinin did not induce interferon. The antigen responsible for stimulating the lymphocyte response and interferon induction is a cross-reactive determinant present on all human and non-human influenza viruses tested.

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