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Immune Interferon Induced by Phytohemagglutinin in Nude Mouse Spleen Cells

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Phytohemagglutinin is able to trigger interferon synthesis in spleen cell cultures from nude (nu/nu) mice as effectively as in splenic cell cultures from haired, control (nu/+), thymus-bearing mice. A minor theta-bearing cell population present in the spleen of nude mice appears essential to phytohemagglutinin interferon production, although cooperating cells are also required. The properties of nude mouse phytohemagglutinin interferon are indistinguishable from those displayed by the interferon induced in thymus-bearing mouse spleen cell cultures. Both interferons are unstable at pH 2 and cannot be neutralized by an antiviral interferon serum; hence, their characteristics correspond to those described for type T interferon. As in the case of viral interferon, pretreatment of L cells with nude phytohemagglutinin interferon induced specific enhanced phosphorylation of a 67,000-molecular-weight protein in vitro when cell extracts were incubated with double-stranded RNA and γ-[^{32}P]ATP.

Interferons are antiviral proteins synthesized by many kinds of somatic cells in response to viral infection. Nonviral substances such as specific antigens and T- and B-lymphocyte stimulants are also able to trigger interferon synthesis but only in immunocompetent cells. These immune-induced interferons, which appear in the course of many immunological events, may play a part in regulating the immune response (11).

We recently reported (29) that interferons induced by nonviral inducers display different antigenic and physicochemical properties, depending on the specificity of the inducer towards lymphocytes. Murine interferons induced by B mitogens (B-type interferon) show some properties similar to those of viral-induced interferon: they are neutralized by antiviral interferon antibodies and are stable at pH 2 (1, 29, 32). Interferons induced by T mitogens (T-type interferon) such as phytohemagglutinin (PHA) (29), concanavalin A (12), or tuberculin (32)—a T-dependent-specific antigen when injected in BCG-sensitized mice—are not neutralized by antiviral interferon serum and are unstable at pH 2.

While studying immune interferon production, we found that PHA induced interferon synthesis in nude mouse spleen cell cultures. Nude mice, homozygous for the recessive gene nu (20), are born without a thymus and are thus severely depleted in thymic-derived lymphocytes (15, 21). They are deficient in T-cell functions, lack T-helper cell activity (2), and are unable to give a graft-versus-host response (16, 31). However, a small percentage of theta-bearing cells and T-cell precursors has been detected in spleen of nude mice (15, 21, 26).

We report here that T-mitogen PHA triggered interferon synthesis in spleen cell cultures from nude mice just as effectively as in similar cultures from normal mice. We also show that a theta-bearing minor cell population present in the spleen of nude mice is essential for PHA interferon production, although cooperating cells are also required. Furthermore, we demonstrate that the properties of this interferon are indistinguishable from those displayed by the interferon induced in thymus-bearing mouse spleen cells by the T mitogen.

MATERIALS AND METHODS

Animals. Usually, 10- to 16-week-old males were used. BALB/c mice were supplied by Charles River (France). Nude mice, homozygous for the *nu* gene (nu/nu) and their (nu/+) heterozygous littermates (haired control mice) were supplied by the Centre de Selection et d'Elevage d'animaux de laboratoire (CSEL), France.

Interferon titrations. Interferon titrations were performed by the cytopathogenic inhibition test in L-cell culture microplates using vesicular stomatitis virus as challenge. Titers were expressed in international reference units (9).

Anti-interferon serum. Anti-mouse L-cell interferon serum was prepared by repeated inoculation of rabbits with semipurified Newcastle disease virus (NDV) interferon (specific activity; 10^7 IU/mg of pro-

tein) for a period of 8 months. The first two injections were of 1×10^6 U of interferon, followed by injections of 1×10^5 interferon U every 4 weeks for 6 months.

Anti-interferon neutralization titer was determined as described by Ogburn et al. (18). Briefly, 10 U of each interferon preparation was mixed with an equal volume of a serial twofold dilution of anti-interferon serum and incubated at 37°C for 60 min. A fraction of each mixture was then assayed for interferon activity. The reciprocal of the dilution of antiserum that permitted the development of 50% of the cytopathogenic effect was considered as the antiserum titer.

Spleen cell cultures and interferon induction. Spleen cells were cultured at a concentration of 5×10^6 cells/ml, in RPMI 1640 (Flow), supplemented with glutamine (2 mM), 5% fetal calf serum (Flow), and 4 mg (%) of gentamicin. For interferon production, purified PHA (Wellcome) was added to 1-ml cultures, unless otherwise indicated, and incubated for 24 h at 37°C in a humidified 5% CO_2 incubator; the cultures were then centrifuged and supernatants were kept at −70°C until interferon titration. For the preparation of large amounts of interferon, 15-ml cell suspensions were incubated in 100-mm diameter petri dishes, as described above. After removing the cells, the culture medium was precipitated at 4°C with ammonium sulfate at 42% saturation, and the supernatant was concentrated 10- to 20-fold under vacuum and dialyzed against phosphate saline. The different types of interferon will be referred to, respectively, as nude mouse PHA interferon and haired control mouse PHA interferon, depending on the origin of the spleen cells.

Fractionation of spleen cells. Depletion of immunoglobulin (Ig)-bearing cells was performed using plastic beads (Degalan V_{26} Degussa; Wolfgang A. G. Hanau am Mair, Germany) coated with mouse Ig anti-mouse Ig, essentially as described by Wigzell (30).

Briefly, beads were coated by incubation with mouse Ig (prepared by ammonium sulfate precipitation of normal mouse serum) at a concentration of 2 mg/ml of beads for 40 min at 45°C and left to stand overnight at 4°C. The Ig-coated beads were transferred into a plastic column (10-ml bed volume) and washed with medium (RPMI 1640, Flow); 2 ml of rabbit anti-mouse Ig, (Pasteur Institute) was then applied, and the column was again washed with RPMI supplemented with 10% fetal calf serum. Approximately 100×10^6 to 130×10^6 cells in this medium were applied to the column, which was eluted with the same medium. Fractionation was carried out at 4°C at a rate of 40 ml/h. Then 6×10^6 to 8×10^6 cells were recovered (about 6% of the cells applied), and they were 95 to 98% viable as judged by the trypan blue exclusion test.

Treatment with anti-theta serum. Anti-theta serum was prepared by the method described by Fournier and Bach (8), i.e., inoculation of AKR mice with thymocytes from CBA mice, and was a gift from J. F. Bach. For depletion of theta-positive spleen cells, suspensions at 5×10^6 cells/ml in RPMI without serum were incubated at 37°C with anti-theta serum (1/50 final dilution). After 15 min, complement was added and incubation prolonged for 1 h. Cells were washed three times with medium before any subsequent treatment. Cytotoxicity of anti-theta serum was determined by the trypan blue exclusion test. The antiserum used was checked to make sure it was active on T cells but not on B cells. Treatment of suspensions of normal mouse spleen cells BALB/c and haired control mouse spleen cells with this anti-theta serum and complement completely abolished the mitogenic response ([^{3}H]thymidine incorporation) to PHA while leaving the lipopolysaccharide response unaffected.

Cell-free extracts and phosphorylation. Confluent mouse L cells grown in petri dishes were incubated with the indicated doses of interferons in Eagle minimal essential medium containing 6% newborn calf serum (Flow). After 24 h at 37°C under 5% CO_2, the petri dishes were rapidly chilled in ice, and the medium was eliminated. Cells were washed twice with cold washing buffer (3.5 mM tris(hydroxymethyl)-aminomethane-hydrochloride [pH 7.5]–140 mM KCl), recovered by scraping with a rubber policeman, and lysed at 4°C by allowing them to stand for 30 min in hypotonic buffer (10 mM HEPES[*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid]-hydrochloride, pH 7.5, 10 mM KCl, 1.5 mM magnesium acetate) containing 0.5% Nonidet P-40 (Shell). Afterwards 10-fold concentrated buffer was added to give a final concentration of 20 mM HEPES-hydrochloride, pH 7.5, 120 mM KCl, 5 mM magnesium acetate, 7 mM 2-mercaptoethanol, and 10% glycerol. Lysates were then centrifuged at $10,000 \times g$ for 10 min. Supernatants (S-10) were filtered by centrifugation through a syringe containing Sephadex G-25 (bed volume = 10 times the lysate volume). Excluded material was then fractionated and stored frozen in liquid nitrogen until used.

Reaction mixture (25 μl) contained 30 mM HEPES, pH 7.5, 7 mM 2-mercaptoethanol, 0.2 mM γ-[^{32}P]ATP (final specific activity, 500 mCi/mM), 0.25 mM GTP, 5 mM creatine phosphate, 3.5 mM magnesium Acetate, 180 μg of creatine phosphokinase per ml, and 0.3 absorbance at 260 nm units of S-10 extracts (usually 20 μl). Where indicated, polyinosinic acid-polycytidylic acid (200 ng/ml) was added. After incubation for 60 min at 32°C, 1.3 μl of pancreatic ribonuclease (5 mg/ml in 10 mM ethylenediaminetetraacetic acid–1 mM sodium phosphate, pH 7.0) was added (33). After 10 min of further incubation, mixtures were analyzed by polyacrylamide-sodium dodecyl sulfate slab gel electrophoresis with a linear 10 to 20% acrylamide gradient and the discontinuous buffer system of Laemmli (13). After staining, destaining, and drying, gels were subjected to autoradiography with Kodak X-Omats 5 X-ray film. Molecular weights were established using the following marker proteins: β-galactosidase 130,000; phosphorylase b, 100,000; bovine serum albumin, 68,000; ovalbumin, 43,000; β-carboxipeptidase, 34,000; and β-lactoglobulin 18,000.

Determination of interferon molecular weight. Molecular weights of PHA interferons were determined by filtration on a calibrated Sephadex G 150 column as described elsewhere (6).

RESULTS

PHA interferon synthesis in spleen cell cultures of nude (nu/nu) and haired control

(nu/+) mice. Unlike spleen cells from haired control mice, spleen cells from nude mice did not show enhancement in [^{3}H]thymidine incorporation into DNA when exposed to PHA. Similar results have already been reported (10, 27). In a typical experiment, when 5×10^6 haired control mouse spleen cells were incubated with 2.5 μg of PHA in 1-ml cultures, [^{3}H]thymidine incorporation into trichloroacetic acid-insoluble material (measured by current technique) increased seven- to ninefold compared to nontreated cultures. No significant increase was observed for nude mouse spleen cell cultures when incubated with PHA in the same conditions. However, PHA induced interferon synthesis in both cases.

Figure 1 gives the PHA dose-response curves for interferon production in splenic cell cultures from nude (nu/nu) and haired control (nu/+) mice. The data given in this figure indicate that both curves are similar within experimental errors. Interferon synthesis by spleen cells from nude mice was dose dependent, the maximum response (250 IU/ml) being obtained with 3.5 to 4 μg of PHA per ml. With haired control mouse spleen cells, or splenic cells from BALB/c mice (not shown), maximum interferon induction (160 IU/ml) was reached with 2.5 to 3 μg with a plateau up to 5 μg of PHA per ml; no significant interferon synthesis (less than 10 IU) was observed without addition of PHA.

The same profile for the dose-response curve was obtained in several independent experiments, although interferon titers differed from

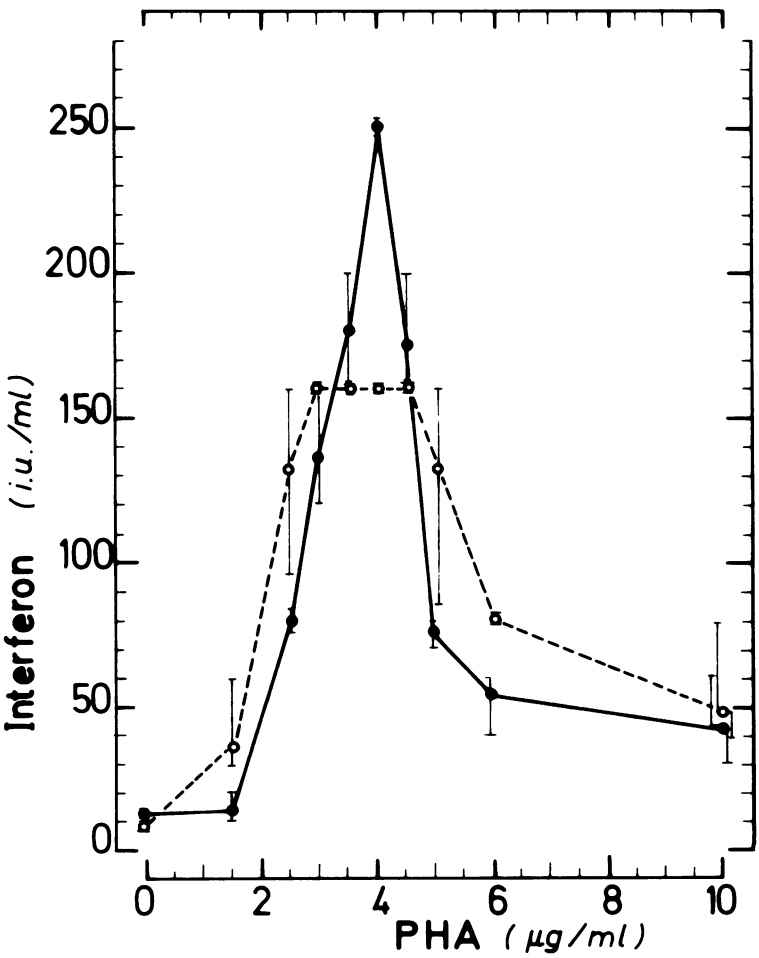

FIG. 1. *PHA dose-response curves of interferon synthesis in splenic cell cultures from nude (nu/nu) and haired control (nu/+) mice. Each point represents the mean and range of interferon titer produced by three individual spleen cell cultures from three nude and three haired control mice. Interferon was induced with different doses of PHA, as described under Materials and Methods. Nude mice (●), haired control mice (○).*

one experiment to another.

When spleen cells were treated with an inhibitor of protein synthesis; cycloheximide, 10 μg/ml, for 3 h before stimulation with PHA, no interferon was detected in the supernatants. The chosen dose of cycloheximide was without toxic effect (data not shown).

Characterization of the antiviral activity induced by PHA in nude mouse spleen cell cultures: antigenic properties and pH stability. Lability to acid pH and absence of neutralization by sera directed against viral interferon are characteristics of interferons induced by T-cell stimulants (called T-type interferon [29], immune interferon [5], or type II interferon [32]). We tried to find out whether PHA interferon synthesized by spleen cells from nude mice displayed the same behavior. Interferon preparations from haired control (nu/+) mice were also included in these assays.

Table 1 shows the results obtained by the in vitro neutralization test using a rabbit serum against NDV L-cell interferon. This antiserum, which has a neutralization titer of 20,000 against 10 NDV L-cell interferon U, was unable to neutralize the antiviral activity induced in nude and haired control mouse spleen cells by PHA. On the other hand, about 80% of both interferon activities (haired control and nude) were destroyed by pH 2 treatment. Their respective molecular weights, estimated on Sephadex G 150, were very similar—36,000 (haired control) and 39,000 (nude). Thus, despite the absence of the thymus, PHA interferon induced in nude mouse spleen cells showed similar properties to those induced in thymus-bearing, haired control mouse spleen cell cultures.

Lymphoid cell type(s) involved in PHA interferon production in nude mice. Less than 5% of theta-bearing cells can be detected in spleen cells from the nude (nu/nu) mice we used (26). As PHA in soluble form is known to activate T cells, we performed several experiments to determine whether the few T cells present in the spleen of these animals were involved in interferon synthesis. As reported by others (10, 27) we confirmed that no thymidine incorporation is observed when these spleen cells are incubated with PHA, whereas haired control (nu/+) mice behave like normal mice. However, as shown in Table 2, no significant interferon production was observed when cultures were pretreated with anti-theta serum and complement before PHA stimulation. Treatment with anti-theta serum alone or complement alone did not affect interferon synthesis. Hence, these results indicate that the minor population of theta-positive cells played an essential part in interferon synthesis. Because synthesis was abrogated by the anti-theta serum pretreatment, we performed affinity chromatography on a mouse Ig anti-mouse Ig Degalan column to obtain the theta-positive cells and find out whether they were the interferon-producer cells. The affinity column retained the Ig-bearing cell population while allowing a cell fraction enriched by theta-bearing lymphocytes to pass through. Only about 6% of cells from the starting cell suspension were recovered after filtration through the column, and at least 60% of the cells recovered were found to be sensitive to the cytotoxic effect of anti-theta serum and complement. The Degalan-excluded cells failed to produce interferon when incubated with PHA. However, as shown in Fig. 2, the interferon titer was enhanced when Degalan-excluded cells were added to the total spleen cell population from nude or BALB/c mice. No significant modification of interferon titers was obtained by adding similar amounts of total splenic cells (not shown). The amount of interferon produced depended on the number of Degalan-excluded cells added.

Phosphorylation of a specific protein. It was recently demonstrated that after pretreatment of cells with viral homologous interferon, enhanced phosphorylation of a 67,000-molecular-weight protein occurred in vitro when cell

TABLE 1. *Antigenic properties, pH stability, and apparent molecular weight of PHA interferons induced in nude (nu/nu) and haired control mice (nu/+) spleen cell cultures compared to NDV L-cell interferon*

Interferon	Antiserum titer against 10 U of interferon	Residual activity after pH 2 treatment (%)	Mol wt
Nude PHA interferon	<40	20	39,000 ± 2,000
Haired control PHA interferon	<40	20	36,000 ± 2,000
NDV L-cell interferon	20,000	100	25,000 ± 2,000

TABLE 2. *Effect of anti-theta serum on PHA interferon induction in nude mouse spleen cell cultures*[a]

Culture treatment[b]			Interferon titer (U/ml)
Anti-theta serum	Complement	PHA[c]	
−	−	−	6
−	−	+	200
+	+	+	0
−	+	+	160
+	−	+	160

[a] 5×10^6 total splenic cells/ml.

[b] Conditions for different treatments are as in Materials and Methods.

[c] 3.5 μg/ml of culture.

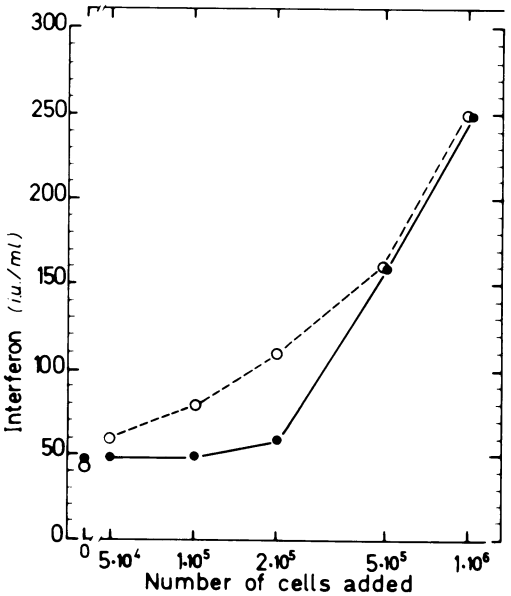

FIG. 2. *Effect of adding Degalan-excluded splenic cells from nude mice, on PHA interferon synthesis. Increasing amounts of Degalan-excluded cells from nude mice were added to 2.5 × 10^6 total splenic cells from nude (●) or BALB/c (○) mice, adjusted to a final volume of 0.5 ml and incubated with 1.15 μg of PHA for 24 h at 37°C in a 5% CO_2 incubator. Interferon was measured in the supernatant as described under Materials and Methods.*

extracts were incubated with double-stranded RNA and γ-[^{32}P]ATP. (14, 22, 33).

The phosphorylated protein can be considered as a biochemical marker of interferon action on the cells.

To see whether PHA interferons induced in spleen cell cultures from nude and haired control mice also displayed this property, we prepared cell-free extracts (S-10) from L cells, pretreated for 24 h with both PHA interferons and also viral interferon. Phosphorylation was performed under the conditions described in Materials and Methods.

Figure 3 gives the autoradiograph of an electrophoresis of extracts from L cells, pretreated or not with viral and PHA interferons and incubated with γ-[^{32}P]ATP in the presence or absence of 200 ng of polyinosinic acid-polycytidylic acid per ml. This figure shows that a phosphorylated band which migrates with an apparent molecular weight of 67,000 is present in the cell-free extracts from cells treated with viral interferon (track 7), nude mouse PHA interferon (track 5), and haired control mouse PHA interferon (track 3). This band is very faint in extracts from untreated cells (track 1). When extracts were incubated in the presence of γ-[^{32}P]ATP and double-stranded RNA, no significant modification was observed in extracts from untreated cells (track 2), whereas strongly enhanced phosphorylation of the 67,000-molecular-weight protein was observed in all three extracts from L cells pretreated with viral interferon (track 8), nude mouse PHA interferon (track 6), and haired control mouse PHA interferon (track 4).

DISCUSSION

The results reported here show that PHA is able to induce interferon synthesis in spleen cell cultures from nude mice. This was a surprising finding since spleen cell populations from nude mice are known to include very few theta-positive (15, 21) cells and do not show enhancement in thymidine incorporation into DNA when stimulated with PHA. In this paper we have described experiments designed to identify the immunocompetent cell or cells involved in interferon synthesis.

PHA interferon synthesis was abolished when spleen cell cultures were pretreated with anti-theta serum and complement. Although less than 5% of theta-bearing cells were present in these cultures (26), the absence of interferon induction when these cells were destroyed indicated that cells from the T lineage were involved in interferon synthesis. Stobo et al. (25) and Wallen et al. (28) reported similar observations for interferon synthesis in normal mouse spleen cells. However, Stobo et al. (25) found that thymocytes, which were nearly pure T cells, were unable to synthesize interferon after PHA stimulation. Neuman and Sorg (17) showed similar results using T cells isolated from normal mouse spleen cells. In our study, the Degalan-excluded cells, constituting a fraction enriched in theta-positive cells (more than 60%) also failed to produce a significant amount of interferon when incubated with PHA. Nevertheless, when increasing amounts of these cells were added to a constant number of total spleen cells from nude or BALB/c mice, higher interferon titers were obtained. Hence this minor theta-positive cell population seems to control interferon production, although cooperation with other cells such as macrophages is probably an absolute requirement. Whether the synthesis of interferon takes place in a cell coming from the theta-positive pool remains to be clarified.

Smith and Eaton (23) recently reported that a subpopulation of spleen cells from nude mice was able to impair lipopolysaccharide stimulation of spleen cells; this property was abrogated by treatment with anti-theta serum and complement: nude mice would therefore have T-func-

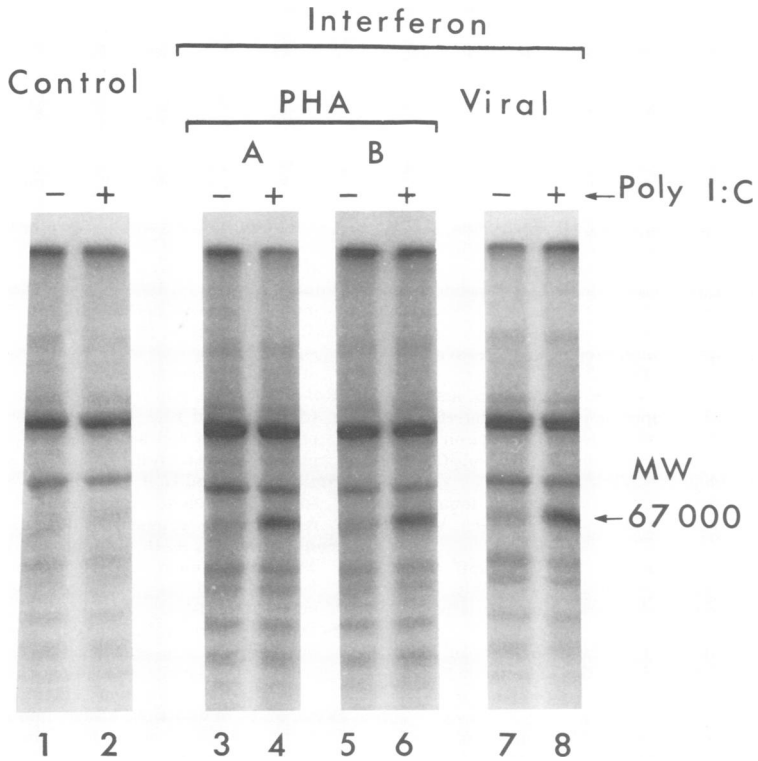

FIG. 3. *Autoradiograph of electrophoresis of phosphorylated proteins from control and interferon-treated cell extracts in the absence and presence of polyinosinic acid-polycytidylic acid Tracks 1 and 2, Untreated cell extracts. PHA interferon-treated cell extracts: (A) haired control mouse interferon; (B) nude mouse interferon; Tracks 7 and 8, NDV L-cell interferon-treated cell extracts. Doses of interferon were 80 IU/ml, with (+) or without (−) polyinosinic acid-polycytidylic acid.*

tional suppressor cells. On the other hand, evidence has been given suggesting that interferon plays a part in the mediation of suppressor T-cell effects (3, 4, 12, 14, 24). It is conceivable that PHA activates the T-suppressor cells or precursors of these cells present in nude mice, thus triggering the interferon synthesizing system.

Active cell populations present in nude mice involved in interferon synthesis might be similar to those present in normal mice, since titer levels for the respective interferon preparations are of the same order.

We compared certain properties of the interferons produced by PHA stimulation of spleen cell cultures from nude and haired control mice, respectively. They were not neutralized by anti-mouse NDV L-cell interferon, were unstable at pH 2, and had similar molecular weights. Hence, nude mouse PHA interferon responds to the characteristics of T-type interferon (29). To our knowledge this is the first report showing the synthesis of a T-mediated lymphokine in nude mouse spleen cell cultures. Regarding the antigenic properties and pH stability, these T-type interferons are different from viral interferon. However, they induced phosphorylation of a 67,000-molecular-weight protein in L cells pretreated with them. When these L-cell extracts were supplemented with double-stranded RNA, phosphorylation was enhanced to the same degree as for viral interferon. This phosphorylated protein can be considered as a marker of the interferon action (14, 22, 33). The role of the 67,000-molecular-weight protein is still unclear, but on the basis of results reported in the rabbit reticulocyte system (7), it would be a kinase which phosphorylates one of the protein synthesis initiation factors and in this way inhibits translation. PHA interferon and NDV L-cell interferon are different molecules. Nevertheless, in both cases, the establishment of the antiviral state requires novo synthesis of RNA and proteins (29), and both interferons induce the same phosphorylation pattern in L-cells, suggesting that they act by a similar antiviral mechanism.

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