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Failure of Athymic-Nude Mice Sensitized with *Bacillus Calmette–Guérin* to Produce Interferon in Response to Purified Protein Derivative (41556)

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Abstract. Unlike their +/+ and nu/+ littermates, homozygous athymic-nude (nu/nu) mice that had been sensitized with *Bacillus Calmette–Guérin* (BCG) failed to produce interferon (IFN) in response to purified protein derivative (PPD). Likewise, spleen cells derived from BCG-sensitized nu/nu mice failed to produce IFN when exposed to PPD *in vitro*. These findings suggest that the T-cell subset that responds to specific antigens (i.e., PPD) is absent in athymic-nude mice.

The production of immune (or type II) interferon (IFN-γ)[3] has been described in various experimental systems, such as spleen cells induced with the T-cell mitogens concanavalin A (Con A), phytohemagglutinin (PHA), or staphylococcal enterotoxin A (SEA); lymphoid cells treated with anti-lymphocyte serum or galactose oxidase; primary and secondary mixed lymphocyte cultures (MLC); and, finally, sensitized cells upon reexposure to the specific antigen (for a review see (1)).

The induction of IFN-γ depends on the cooperation of different cell types, i.e., T and B lymphocytes and macrophages (2), although the relative contribution of these different cell types in the induction process has not been clearly assessed. Moreover, cloned T cells are by themselves capable of producing IFN-γ when exposed to Con A or specific antigens (3).

Spleen cell cultures derived from athymic-nude (nu/nu) mice produce interferon in response to PHA or SEA (4, 5) but fail to do so when stimulated by either allogeneic cells or Con A (6, 7). It is likely that the subpopulation of T cells that normally responds to Con A or alloantigens is absent in nu/nu mice (or nu/nu mouse spleen cells) (7). This contention may also hold for specific antigens, since, as described in the present report, nu/nu mice which have been immunized with BCG fail to produce interferon in response to PPD under conditions where their heterozygous littermates and homozygous +/+ counterparts are able to mount considerable interferon yields.

1. Materials and Methods. *1.1 Mice.* Outbred NMRI (Naval Medical Research Institute) nu/nu, nu/+, and +/+ mice were reared and maintained as described before (8, 9). C57Bl/6J BOM nu/nu, nu/+, and +/+ mice were obtained from G-I Bomholtgård Ltd., Ry, Denmark. Mice weighing 20–25 g were used throughout all experiments.

1.2 Lymphoproliferation assay. Unfractionated spleen cells were suspended at 5×10^5 cells/ml in RPMI 1640 medium, supplemented with 5% FCS (fetal calf serum), antibiotics, and 5×10^{-5} *M* 2-mercaptoethanol. The cells were plated in flat-bottomed microplates (Sterilin) in 200-μl volumes. To activate the lymphocytes, 20 μl of freshly prepared mitogen solutions were added per well. Final concentrations of the mitogens were: Con A (concanavalin A, Pharmacia), 4 μg/ml; PHA (phytohemagglutinin, Wellcome), 15 μg/ml, and LPS (lipopolysaccharide *E. coli* 055B5, Instituut Pasteur Brabant), 20 μg/ml. The cells were cultured for 72 hr in a humidified CO_2 incubator, pulse labeled with 0.4

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[3] Abbreviations used: BCG, *Bacillus Calmette–Guérin*; PPD, purified protein derivative; PHA, phytohemagglutinin; Con A, concanavalin A; SEA, staphyloccocal enterotoxin A; MLC, mixed lymphocyte culture; VSV, vesicular stomatitis virus; NDV, Newcastle disease virus; MuIFN-α,β, murine type I interferon; MuIFN-γ, murine type II (immune) interferon; PBS, phosphate-buffered saline.

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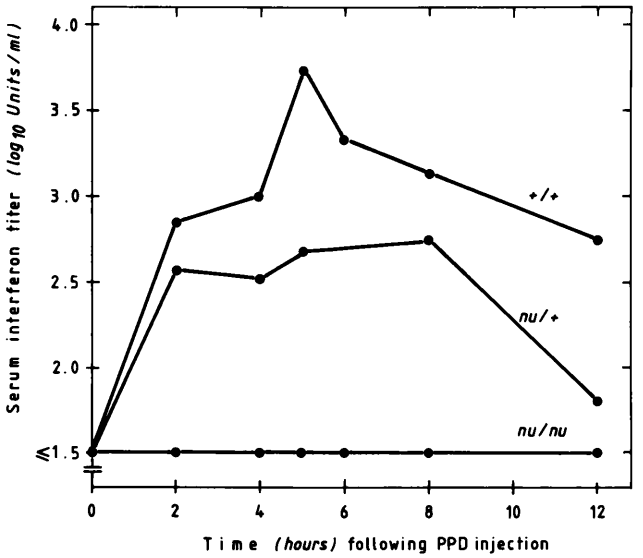

FIG. 1. Serum interferon titers of NMRI nu/nu, nu/+, and +/+ mice immunized with BCG and challenged 2 weeks later with PPD. The interferon titers were determined at different times after PPD injection on the pooled serum fractions of three mice and are expressed in laboratory MuIFN-γ units; 0.6 log unit should be added to each value to obtain the interferon titers in terms of the National Institute of Health (USA) MuIFN-α, β standard G002-904-511.

μCi [*methyl*-^{3}H]thymidine (Amersham, 8.3 mCi/mg) per well and harvested 5 hr later on a Titertek Cell Harvester (Skatron). The radioactivity recovered from the filters was measured in a Beckman LS.230 liquid scintillation counter. All experiments were done in quadruplate. Results are expressed as the mean ± SD counts per minute (cpm).

1.3. Interferon production. 1.3.1. Antigen-induced IFN. The mice were sensitized intravenously with BCG (BCG strain GL 2, grown as surface pellicle) at 5×10^6 bacteria/mg/0.2 ml/mouse (or PBS (phoshate-buffered saline) at 0.2 ml/mouse) and 2 weeks later injected intravenously with PPD at 1 mg/0.2 ml/mouse (or PBS at 0.2 ml/mouse). Blood samples were taken at several times (2, 4, 5, 6, 8, and 12 hr after the injection of PPD (or PBS) and the sera were pooled (three mice/group).

For the *in vitro* production of IFN-γ, mice were killed by cervical dislocation 2 weeks after the BCG inoculation. Spleens were removed aseptically, and the cells were dispersed in a loosely fitting Dounce homogenizer, counted in a Coulter counter, and suspended at 5×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FCS, antibiotics, and 5×10^{-5} *M* 2-mercaptoethanol. PPD was added at various doses and aliquots of the culture supernatant were harvested for IFN titration at either Day 1, 2, 3, or 4.

1.3.2. Mitogen-induced IFN. Spleen cells derived from untreated mice were cultured as described in section 1.3.1. The cells were stimulated by Con A (4 μl/ml) or PHA (15 μl/ml) and culture supernatants were harvested for IFN titration on Days 1, 2, 3, and 4.

1.4. Interferon assay. Serum interferon titers were determined by a virus plaque reduction assay in mouse L-929 cells using VSV as the challenge virus. A laboratory standard of mouse type I interferon (MuIFN-α,β), calibrated against the National Institute of Health (USA) mouse type I interferon standard G002-904-511, was included in the interferon assays and gave a titer ($10^{2.9}$ units/ml) that was 0.6 × log unit lower than the expected titer ($10^{3.5}$ units/ml).

2. Results. *2.1. In vivo production of antigen-induced IFN.* As shown in Fig. 1, NMRI +/+ mice which had been immunized with BCG produced significant amounts of MuIFN-γ after PPD injection (up to about 5000 units/ml at the peak of the interferon

response). Their homozygous nu/nu counterparts were completely unresponsive to the PPD challenge. In the heterozygous nu/+ mice intermediate interferon levels were obtained. This indicates that the production of PPD-induced interferon (in BCG-sensitized mice) is under the control of a codominant gene, perhaps expressed only in mature T cells.

The kinetics of appearance of MuIFN-γ in the +/+ mice was similar to that described by Stinebring and Absher (10): thus, the serum interferon titer reached its peak value at 4 to 6 hr after PPD injection and declined to about 10% of its peak value at 12 hr after PPD injection. The data presented in Fig. 1 concern the interferon titers obtained upon a double course of events (BCG infection followed 2 weeks later by a PPD injection). No interferon was detected in the bloodstream of +/+, nu/+, or nu/nu mice which were given BCG followed by PBS or PBS followed by PPD.

2.2 In vitro production of antigen-induced IFN. As shown in Fig. 2, spleen cells from C57Bl/6J +/+ mice, previously immunized with BCG, produced significant amounts of interferon following *in vitro* exposure to PPD. The highest IFN titers were obtained with PPD at 150 and 500 μg/ml (the highest concentrations tested) but even at lower PPD concentrations significant IFN levels were noted. In keeping with the data obtained *in vivo* (section 3.1), homozygous nu/nu spleen cells were totally refractory to the interferon inducing effects of PPD. The heterozygous nu/+ spleen cells produced intermediate interferon levels. Spleen cells derived from nonimmunized +/+ mice did not produce IFN in response to PPD; neither did spleen cells from BCG-immunized mice in the absence of PPD (data not shown).

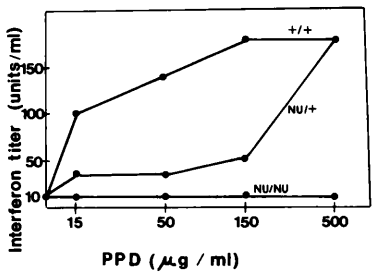

FIG. 2. Interferon titers obtained in the supernatant of spleen cell cultures derived from C57Bl/6J BOM nu/nu, nu/+, and +/+ mice immunized with BCG and exposed to PPD *in vitro*. The spleen cells were removed 2 weeks after immunization of the mice with BCG and incubated with PPD for 24 hr. The interferon titers were determined as a function of the PPD concentration and are expressed in laboratory MuIFN-γ units as explained in the legend to Fig. 1.

TABLE I. INTERFERON PRODUCTION IN SPLEEN CELL CULTURES DERIVED FROM NMRI nu/nu AND +/+ MICE

Inducer[a]	Cell source[b]	Interferon titer[c] 24 hr	48 hr	72 hr	96 hr
PPD	+/+	178	178	178	178
	nu/nu	<10	<10	<10	<10
Con A	+/+	100	56	18	18
	nu/nu	<10	<10	<10	<10
PHA	+/+	178	100	56	18
	nu/nu	<10	<10	<10	<10
NDV	+/+	178	178	178	178
	nu/nu	18	32	32	25

[a] PPD, 500 μg/ml; Con A, 4 μg/ml; PHA, 40 μg/ml; and NDV 100 hemagglutination units/ml.
[b] Used at 5 × 10^6 spleen cells/ml (derived from mice sensitized with BCG 2 weeks before PPD challenge; not sensitized if used with Con A, PHA or NDV as challenge).
[c] Expressed in laboratory units (see legend to Fig. 1).

2.3 Reactivity of nu/nu mouse spleen cells to T-cell mitogens in vitro. Table I presents the kinetics of the IFN production in cultures of 5 × 10^6 spleen cells/ml activated with PPD, Con A, PHA, or NDV (Newcastle Disease virus (strain La Sota), inactivated by β-propiolactone). BCG-sensitized +/+ spleen cells produced substantial amounts of interferon in response to PPD. This interferon production remained constant for at least 4 days. However, BCG-sensitized nu/nu spleen cells failed to produce IFN in response to PPD, irrespective of the time of incubation (1, 2, 3, or 4 days).

Although other authors have reported that PHA is capable of inducing interferon in nude mice (4), we were unable to demonstrate interferon induction by PHA in nu/nu mouse spleen cells, whether these cells were derived from NMRI nu/nu mice (Table I) or C57Bl/6J BOM nu/nu mice (data not shown). Con A also failed to induce IFN in nu/nu mouse spleen cells, which is consistent with the observations of Kirchner *et al.* (7). In contrast with the nu/nu mouse spleen cells, nu/+ and +/+ mouse spleen cells produced significant

TABLE II. PROLIFERATION OF SPLEEN CELLS DERIVED FROM nu/nu, nu/+, AND +/+ MICE

Cell source	Inducer				
	None[a,c]	PPD[a,b]	Con A[a,c]	PHA[a,c]	LPS[a,c]
nu/nu	1570 ± 244	20,705 ± 451	1,068 ± 170	385 ± 28	31,995 ± 2350
nu/+	1530 ± 106	18,470 ± 798	69,330 ± 2595	32,295 ± 2036	65,715 ± 3775
+/+	1510 ± 133	8,830 ± 410	107,615 ± 6805	56,725 ± 4020	69,740 ± 3140

[a] The values are expressed as the mean ± SD radioactivity counts (cpm) obtained for quadruplate cell cultures.
[b] Spleen cells derived from C57Bl/6J BOM mice, sensitized 2 weeks earlier with BCG and incubated *in vitro* with PPD (500 μg/ml) for 24 hr; [*methyl*-^{3}H]thymidine was added during the last 5 hr of this 24-hr incubation period.
[c] Spleen cells derived from NMRI mice and stimulated *in vitro* with Con A, PHA, or LPS as described in section 2.2; [*methyl*-^{3}H]thymidine was added during the final 5 hr of the 3-day incubation period.

amounts of interferon in response to either Con A or PHA. The IFN titers induced by Con A and PHA reached their peak value at 24 hr and declined during the next 3 days. These IFN levels were comparable to those described in the literature.

Although unresponsive to the interferon-inducing activity of PPD, Con A, and PHA, nu/nu mouse spleen cells produced appreciable amounts of IFN upon exposure to NDV. However, these IFN yields were significantly lower than those obtained with nu/+ or +/+ mouse spleen cells.

As shown in Table II, nu/nu mouse spleen cells did not proliferate upon stimulation with the T-cell mitogens Con A and PHA. The B-cell mitogen LPS caused a significant proliferative response in nu/nu mouse spleen cell cultures, albeit to a lesser extent than in nu/+ and +/+ spleen cell cultures. It is noteworthy that BCG-sensitized nu/nu spleen cells showed a more pronounced proliferative reaction to PPD than the corresponding wild-type +/+ spleen cells. The diminished +/+ spleen cell response may possibly be related to the growth-inhibitory effect of the interferon produced by these cultures.

2.4 Characterization of the antigen- and mitogen-induced interferons. The interferons that were produced by BCG-immunized +/+ mice and +/+ mouse spleen cells in response to PPD were characterized as authentic MuIFN-γ by their sensitivity to pH 2.0 (complete loss of interferon activity after dialysis against 0.1 *M* glycine–HCl, pH 2.0, for 24 hr), relative resistance to heating at 56° for 30 min (only 0.5 log decrease in interferon activity), neutralization by specific antibodies (raised against SEA-induced MuIFN-γ (11)), and lack of neutralization by anti-MuIFN-α,β serum. Mitogen-induced IFN was only partially destroyed by pH 2.0 treatment (residual interferon activity 20 to 30%). It was neutralized by specific antibodies to MuIFN-γ, but not by antibodies to MuIFN-α,β.

3. Discussion. Whereas PHA (4) and SEA (5) are capable of inducing IFN-γ in nu/nu mouse spleen cell cultures, Con A and alloantigens are not able to do so (6, 7). A failure to induce IFN-γ in athymic-nude mice has also been noted with the bacterial immunopotentiator OK-432 (12). As further documented in the present paper, specific antigens, i.e., PPD, also fail to induce IFN-γ in BCG-sensitized nu/nu mice.

Recent studies of mitogen-induced DNA synthesis and interferon production in cells from different lymphoid tissues have indicated that certain mitogens such as PHA, SEA, and Con A selectively activate T-cell subpopulations (13). In view of the present data on interferon induction by specific antigens in nude mice and nude mouse spleen cells and the previously published data on interferon induction by PHA, SEA, Con A, and alloantigens in nude mouse spleen cells, one may postulate that the T-cell subset(s) responding to PHA and SEA are present in nude mouse spleen, whereas the subset(s) responsive to Con A, alloantigens, or specific antigens are not. However, some caution is warranted in the interpretation of the aforementioned data. Indeed, IFN-γ preparations obtained with SEA or PHA as the inducer may also contain IFN-α (14) and Con A-induced IFN-γ may be contaminated by IFN-β (15). Furthermore, the contention that nude mouse spleen cells are able to produce IFN in response to PHA (4) should be reevaluated since we were unable to obtain IFN production with PHA in spleen

cell cultures derived from nude mice of different genetic background (NRMI and C57Bl/6J).

The patterns of production of type I and type II interferon are clearly differentiated. While unresponsive to specific antigens (i.e., PPD), nude mice produce substantial amounts of interferon in response to type I interferon inducers, such as NDV (this report) and polyinosinic acid·polycytidylic acid (9). Moreover, macrophages derived from nude mice infected with BCG or *Corynebacterium parvum* produce similar type I interferon levels as macrophages derived from their normal littermates (16).

Although antibodies raised against SEA-induced interferon can neutralize different immune interferons (induced by PHA, Con A, MLC, and specific antigens) (5), this does not necessarily imply that they are all identical. The diversity of the induction systems and the heterogeneity observed upon purification (17) could indicate that they are not. For human leukocyte interferon there exist at least 12 different genes (18) and the products of these genes show important functional differences, for example in host-range (species specificity). A similar situation may also occur with IFN-γ.

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