

Effect of Niridazole on Mitogen Induced Lymphocyte Proliferation (40454)

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The antischistosomal drug niridazole has been shown to suppress a variety of cell-mediated immune reactions. The *in vivo* reactions which have been suppressed include the tuberculin reaction (1), the development of granulomatous formation around schistosome eggs (2), experimental allergic encephalitis (3), and graft rejection (4). *In vitro* immune reactions such as the production of migration inhibition factor (5) and the mixed lymphocyte reaction (6) were also suppressed. The drug apparently leaves the humoral immune system intact (7). Niridazole itself is responsible for the antischistosomal activity (8) but evidence suggests that metabolites of niridazole are responsible for the immunosuppressive effects (5). These metabolites bind to plasma proteins and remain in circulation after niridazole itself has been eliminated (9). Daniels *et al.* (5) have demonstrated that serum from niridazole-fed guinea pigs will inhibit MIF production by antigen-stimulated lymphocytes. It was observed that MIF production was not suppressed if the lymphocytes were exposed to the antigen for 60 sec prior to the addition of this niridazole activated serum (NAS). Apparently, once a sensitized lymphocyte has been exposed to specific antigen for a short time, changes in the cell occur and it becomes unresponsive to the suppressive effects of NAS. Macrophages exposed to NAS did not lose the ability to respond to MIF nor did they cease to function as antigen processing cells in the primary immune response. Thus, the target cell of NAS appears to be the lymphocyte. The present study was undertaken to determine if mitogen-induced activation of lymphocytes could also be blocked by NAS.

Materials and methods. Powdered niridazole (batch #B75-8 or #B77-19 Ciba-Geigy) was suspended in 3 ml of water and given orally to Hartley guinea pigs at a dose rate of 100 mg/kg. In three days the guinea pigs were bled by cardiac puncture and the NAS

was separated and saved. In a second group of guinea pigs all four footpads were injected with 0.05 ml of complete Freund's Adjuvant (Difco Laboratories) per footpad. In 7 days, the lymph nodes were removed from the inguinal, the axillary, and the cervical regions. The nodes were teased apart in Hank's Balanced Salt Solution (HBSS) and the cells were collected and centrifuged at 200g. The cells were washed a total of three times in HBSS. The exclusion of trypan blue dye was used as an indication of viability.

Microtiter plates of the round-bottom type (Falcon Plastic) were used to assay for mitogenesis. To each well was added 0.05 ml of mitogen, 0.10 ml of either 30% NAS or 30% normal guinea pig serum (NGPS) and finally 0.05 ml from a suspension of 4.0×10^6 lymphocytes per ml. All reagents used in the mitogenesis experiment were prepared in RPMI 1640 (Microbiological Associates) containing streptomycin and penicillin. Bacto Phytohemagglutinin-P (PHA) (Difco Laboratories, lot #3110-56) was tried at concentrations of 1.0, 2.0, 5.0, and 10.0 $\mu\text{g/ml}$ with 2.0 $\mu\text{g/ml}$ yielding the most consistent response in this system. Therefore, a concentration of 2.0 $\mu\text{g/ml}$ was used throughout these experiments. Upon addition of the reagents, the cells were incubated for 48 hr at 37° in 5% CO₂ and 95% humidity. Tritiated thymidine (Schwarz/Mann) was then added to a final concentration of 25.0 $\mu\text{Ci/ml}$ for a 6 hr pulse and the cells were harvested using a multiple automated sample harvester. The radioactivity which had been incorporated into the cells during the radioactive pulse was measured using a scintillation counter.

Results. Response in normal guinea pig serum. Sera from several normal guinea pigs were studied for their ability to support PHA-induced mitogenesis of lymphocytes from a single guinea pig. Triplicate wells were used for each condition. A wide range of responsiveness was observed, as seen in Table I. The

TABLE I. VARIATION IN MITOGENESIS OF PHA STIMULATED GUINEA PIG LYMPHOCYTES IN PRESENCE OF NORMAL GUINEA PIG SERUM (15%).

cpm [³ H-Thymidine incorporation] ^a			
Serum source varied ^b		Cell source varied ^c	
without PHA	PHA 2 µg/ml	without PHA	PHA 2.0 µg/ml
121	1090	49	1,691
141	3692	313	3,021
189	3845	50	4,374
127	6618	39	5,072
165	8067	261	12,744
141	14,104	69	18,486
		980	51,481

^a After incubation for 48 hr in presence of PHA cultures were pulsed for 6 hrs with [³H]thymidine 25.0 µCi/ml.

^b Lymphocytes from a single guinea pig and serum from several different guinea pigs.

^c Lymphocytes from several guinea pigs and serum from a single guinea pig.

counts per minute ranged from a low of 1090 to a high of 14,104. No counts were less than 1000 per min. Table I also shows the results when a single serum was used and the lymph nodes were taken from several different guinea pigs. The responsiveness of the lymphocytes demonstrated a wide degree of variability. Once again, no counts were less than 1000 per min.

In order to determine if sensitization by complete Freund's Adjuvant (CFA) might be contributing to the variation of PHA induced mitogenesis a group of normal guinea pigs was used as a source of lymph node cells. These cells demonstrated at least as much variability in response to 2.0 µg of PHA (4525-41,329 cpm) as did lymph node cells from animals sensitized to CFA.

Response in presence of NAS. Over a period of time, several experiments were performed where lymph node lymphocytes were exposed to PHA in the presence of different NAS. In each experiment, the cells were also exposed to PHA in the presence of normal guinea pig serum (NGPS). Table II shows the data from experiments performed with an NAS which had suppressive activity. The cells were cultured in either 15% normal guinea pig serum or 15% NAS. In the absence of PHA no mitogenesis occurred as shown in column 1 and column 3. When PHA was present, only normal guinea pig serum permitted the mi-

togenic response to occur. Examination of cell viability by trypan blue dye exclusion demonstrated that cells cultured in the presence of NAS were no less viable than those cultured in the presence of normal guinea pig serum.

It was observed that not all NAS were effective in blocking mitogenesis. At this point approximately 40% of the guinea pigs given niridazole did not produce suppressive NAS. The upper part of Table III shows the effects of six NAS, each of which demonstrated some suppressive activity. Only the response to PHA in the presence of NAS is shown. The first column records the degree of proliferation, represented in cpm, supported by NAS #15. In six out of seven experiments this NAS was highly suppressive. The NAS listed on the left of the table were the most suppressive. Those on the right were least effective. NAS #3 was effective in only 2 out of 8 experiments. As one compares the mitogenesis induced in the presence of NAS to the mitogenesis induced in the presence of NGPS (bottom half of Table III) one sees that 53% of the time, the active NAS sera reduced the cpm to less than 1000, and 29% of the time, to less than 500, and that NGPS had no such suppressive capabilities. The differences between the suppression produced by active NAS and the response elicited in the presence of NGPS are illustrated in Fig. 1. Each pair of bars represents a single experiment. The difference between the effect of NAS and NGPS is evident. Statistical analysis comparing the data from NAS-supported cultures to the data from NGPS-supported cultures showed that the differences

TABLE II. SEVERAL EXPERIMENTS COMPARING PHA INDUCED LYMPHOCYTE MITOGENESIS IN THE PRESENCE OF NGPS TO MITOGENESIS IN THE PRESENCE OF AN ACTIVE NAS.

Experiment #	cpm [³ H-Thymidine incorporation]			
	NGPS		NAS ₁₅	
	without PHA	PHA 2.0 µg/ml	without PHA	PHA 2.0 µg/ml
1	220	11,140	47	130
2	118	26,199	59	162
3	163	9259	55	235
4	274	8719	361	364
5	181	23,696	249	446

TABLE III. PHA—INDUCED LYMPHOCYTE TRANSFORMATION IN NAS COMPARED TO PHA—INDUCED TRANSFORMATION IN NGPS.^a

cpm [³ H-Thymidine incorporation]						
NAS #15	NAS #16	NAS #1	NAS #4	NAS #5	NAS #3	
130 ^b	227	78	368	431	298	
162	300	93	509	966	992	
166	526	521	989	1290	3067	
235	620	657	1040	2011	5722	
364	715	781	1063	2847	9324	
446	777	812	1083	5642	14,080	
1110	1407	2332	1481	6944	24,196	
NGPS #1	NGPS #3	NGPS #3	NGPS #4	NGPS #5	NGPS #6	NGPS #7
1691	1520	3090	11,825	5058	2571	3734
3021	6708	5217	13,542	8067	3692	3845
4347	7411	6618	23,696			
5072	14,104					
12,744					NGPS #8	NGPS #9
						NGPS #10
18,486					3759	8764
51,481					4193	11,154
						1090
						2871

^a Both NAS and NGPS present at concentration of 15%.

^b Data listed in order of responsiveness for sake of clarity.

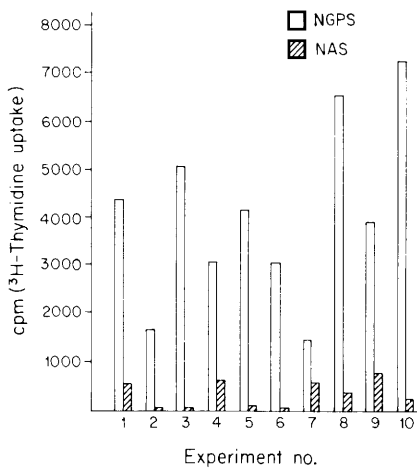


FIG. 1. Lymphocyte transformation induced by PHA. Lymphocytes were exposed to PHA (2.0 μ g/ml) in the presence of either NGPS (15%) or NAS (15%). After 48 hr of incubation [³H]thymidine was added to a concentration of 25.0 μ Ci/ml for 6 hr.

were significant with a probability between .005 and .001.

In an effort to increase the incidence of active NAS, the dose of Niridazole was doubled to 200 mg/kg. The animals were bled in three days. A second group of guinea pigs was given a normal dose of Niridazole but the serum was collected at time intervals of 24, 48, 72, and 96 hr. Neither of these proce-

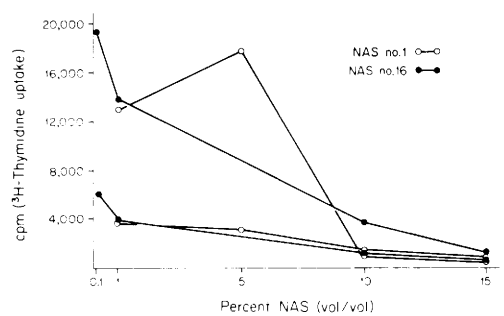


FIG. 2. Mitogenic response of lymphocytes to PHA using increasing concentrations of NAS. PHA concentration was constant at 2.0 μ g/ml. NAS #1 and NAS #16 were each investigated twice. Each line represents a separate experiment.

dures resulted in an increase in the number of active NAS.

Dose response studies. When a range of concentrations of NAS was used in five experiments, the suppressive effect increased as the concentration of NAS increased (Fig. 2). Concentrations of 0.1% and 1.0% were not effective. Concentrations of 10% and 15% yielded maximum suppression. In each case, as NAS was diluted, the lack of such serum was compensated for by the addition of the appropriate amount of NGPS.

Concanavalin-A stimulation. Preliminary work using Concanavalin-A (Con-A) as a stimulant indicated that proliferation induced

by this mitogen can also be inhibited by NAS. In two experiments where 5.0 $\mu\text{g}/\text{ml}$ of Con-A was used to stimulate lymphocytes, NAS reduced the cpm from 4515 and 2313 to 507 and 37, respectively.

Discussion. Several variables became apparent as this model of mitogen induced proliferation was investigated. First, a wide range of responsiveness was seen when lymphocytes from a single source were stimulated with PHA in the presence of different normal guinea pig sera. Second, lymphocytes from different guinea pigs did not uniformly respond when exposed to PHA stimulation even though the same NGPS was used each time. These observations correspond to those reported by other investigators for the mitogenic activation of guinea pig lymphocytes (10). Two additional variables were seen in this particular system, i.e. not all niridazole-fed guinea pigs yielded suppressive serum, and, any single NAS was not suppressive 100% of the time. Faced with this degree of variability one might ask if NAS-induced suppression is only part of the normal variation seen within the system. However, the NAS having suppressive activity reduced the cpm to less than 1000 in 50% of the cases studied. This is compared with NGPS, in which the cpm were consistently greater than 1000. Additionally, dose response studies indicated that decreasing the concentration of NAS decreased the suppression of mitogenesis. This evidence suggests that products of niridazole which are present in NAS can inhibit mitogen-induced lymphocyte proliferation, and that a pathway common to both antigen-activation (5) and mitogen-activation of lymphocytes is blocked by NAS.

It is not clear whether the problem in obtaining consistently active NAS is of a bio-

logic or technical nature. As we attempt to prepare a supply of active NAS adequate to perform kinetic and mechanism studies, it is hoped that information regarding the reasons for these differences might also be forthcoming.

Summary. In previous investigations niridazole has been shown to inhibit manifestations of cell mediated immunity. Serum from niridazole-fed guinea pigs also inhibited the *in vitro* production of MIF by antigen stimulated lymphocytes. We have now presented evidence showing that this same serum can markedly reduce the uptake of [^3H]thymidine by PHA stimulated lymphocytes. Dose response studies demonstrated that the higher the concentration of suppressive serum the stronger was the suppression.

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