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Lymphocyte transformation with bacterial antigens in intrinsic asthma

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Chronic infection of the respiratory tract is often thought to play an important role in intrinsic asthma. To study this further, the response of lymphocytes from patients with intrinsic asthma was investigated by in vitro challenge with various bacterial antigens and assayed by liquid scintillation counting of ^3H -thymidine uptake. Lymphocytes from normal individuals were run in an identical manner for comparison. The mean of the unstimulated background counts from all patients with intrinsic asthma was 250 per cent above the mean of the normal control values. Upon challenge with mixed bacterial antigen, the majority of both patients and control subjects had a 300 per cent or greater increase in counts. Response to H. influenzae antigen seemed to be dose related, with some suppression of ^3H -thymidine uptake at high antigen concentration. It would appear that lymphocytes from intrinsic asthmatic patients respond to challenge with mixed bacterial antigens and also H. influenzae antigen in the same manner and degree as do controls. However, lymphocytes from some patients with intrinsic asthma seem to have a significantly higher unstimulated rate of transformation than do those from normal control subjects. The reasons for this are unknown, and further investigation is indicated.

In 1931, Rackemann¹ proposed the term "intrinsic asthma" for a bronchospastic condition in which neither an allergen nor, in fact, any extrinsic causation can be demonstrated. Various authors²⁻⁴ have used the term "intrinsic" synonymously with infectious, implying that a bacterial infection was the

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etiologic or precipitating event for the asthmatic episodes. Feinberg and associates⁵ suggested that it may not be the organisms themselves but rather metabolic tissue alterations resulting from such infections which are truly responsible for the disease process. Although staphylococcal and pneumococcal infections have been implicated by some,^{6, 7} *H. influenzae* is the organism most frequently suspected because of its prevalence in the sputum of some asthmatic patients.^{2, 8, 9} The significance of these findings is not clear.^{2, 3} There is the possibility that a chronic subclinical infection, although difficult to demonstrate, may produce a continuous challenge to the patient with intrinsic asthma. One consequence of this challenge could be an alteration of the cellular immune response upon re-exposure to the appropriate microbial antigen. Several investigators¹⁰⁻¹² have shown that lymphocytes from individuals rendered hypersensitive by immunization against selected bacteria will undergo increased blast transformation when subsequently challenged with that specific bacterial antigen. Intrigued by the possibility of an altered cellular immune response in intrinsic asthma, the authors chose to investigate this problem by studying the in vitro stimulation of lymphocytes with various bacterial antigens.

METHODS

The subjects for study were 16 adult patients who have chronic bronchial asthma for which convincing extrinsic causes could not be found. In none of these patients was there overt evidence of current bacterial infection, as evidenced by normal white blood counts, normal temperatures, and absence of pulmonary infiltrates in the chest roentgenograms. The majority had mild eosinophilia ranging from 6 to 19 per cent. Stools were negative for ova and parasites. At the time of the study, 5 of the 16 patients were on corticosteroid therapy, receiving between 10 and 30 mg. of prednisone daily. None of the patients was on antibiotic therapy. All patients had decreases in forced vital capacity (FVC) and first-second vital capacity (FEV₁) with improvement greater than 200 ml. after isoproterenol, but diffusing capacity as measured by the single-breath CO technique was within normal limits. Eleven healthy adults without histories of asthma, rhinitis, or other respiratory or allergic disease served as normal controls.

Lymphocytes were obtained from all individuals by dextran sedimentation of heparinized venous blood as follows: 20 ml. of blood was withdrawn into a disposable plastic syringe containing 2,000 units of heparin (Abbott Panheparin) and 4 ml. of 6 per cent dextran (molecular weight 75,000). After 2 hours of sedimentation, the leukocyte-rich plasma was centrifuged for 10 minutes at 4° C., and the cells were washed once and resuspended in Earle's base tissue culture medium 199 with 10 per cent fetal calf serum. Reaction tubes were prepared by adding one million lymphocytes to each culture tube containing 1 ml. of Earle's base tissue culture medium 199 with one per cent "Hepes" buffer, 50 units of penicillin, 50 µg of streptomycin, and 10 per cent fetal calf serum, according to the technique of Bach.¹³

Mixed bacterial antigen was obtained as commercially prepared Parke, Davis Catarrhalis Vaccine No. 615, which contained 5×10^7 organisms per milliliter each of *Neisseria catarrhalis* and *Klebsiella pneumoniae*, 1×10^8 organisms per milliliter each of *Diplococcus pneumoniae*, beta hemolytic streptococcus, *Corynebacterium diphtheroid*, and *Hemophilus influenzae*, and 5×10^8 organisms per milliliter of *Staphylococcus* (*albus* and *aureus*). This vaccine was subjected to multiple filtrations through a dialysis bag with Hanks balanced salt solution being added continuously. Finally, the residue was resuspended in Hanks solution, the volume being constituted to the original amount.

H. influenzae antigen was prepared by suspending *H. influenzae* organisms in Hanks solution. After an aliquot was removed for colony counting, the suspension was diluted 1:1 with sterile deionized water and subjected to freezing at -80° C. followed by rapid thawing and

subsequent sonication to fragment the bacterial cell walls. The suspension was then lyophilized and resuspended in Hanks solution to a final concentration of 1×10^{10} or 3×10^7 organisms per milliliter. All antigen preparations were checked for sterility by culture, and all were found to be sterile.

Aliquots of lymphocyte suspensions were challenged with 20 lambda of the mixed bacterial antigen or 20 lambda of the various concentrations of *H. influenzae* antigen. Control cultures were also challenged with 20 lambda of phytohemagglutinin (PHA). All challenges were performed in duplicate. Lymphocyte cultures for each individual were also run for unstimulated base-line readings without antigen challenge. All cultures were capped and incubated at 37° C. To obtain a time response curve with the bacterial antigens, representative cultures from control subjects were pulsed on the second, third, and fifth days with 2 μ c of tritiated thymidine and then harvested 24 hours later. Patient cultures were pulsed with 2 μ c of tritiated thymidine on the third day and harvested on the fourth day by precipitation with 5 per cent trichloroacetic acid after 3 $\times$ washing in normal saline. The precipitate was solubilized in 0.3 ml. Nuclear Chicago Solubilizer and subsequently transferred into 15 ml. of PPO-POPOP-toluene scintillation fluid and assayed by liquid scintillation counting in a Packard Tri-Carb liquid scintillation spectrometer. Viability was assayed from representative cultures on Day 4 by 0.4 per cent trypan blue dye exclusion. The viability of our lymphocyte cultures was found to exceed 75 per cent throughout the experiments.

RESULTS

Results are expressed as either counts per minute (c.p.m.) or as the ratio of counts per minute of stimulated versus unstimulated cultures (ratio = stimulated cultures (c.p.m.)/unstimulated cultures (c.p.m.)) The time-response curves of lymphocytes from 3 normal control subjects challenged with mixed bacterial antigens are shown in Fig. 1. All cultures had marked increases in ^{3} H-thymidine uptake at 96 hours. At 144 hours, two of the cultures showed less response, while one had increased. As these data indicated that the greatest increase in thymidine uptake occurred between 72 and 96 hours, 96 hours was the time chosen for harvesting subsequent runs. All normal control cultures challenged with PHA had strong responses as well as moderately strong responses to the mixed bacterial antigen challenge. All subsequent cultures were compared with unstimulated control values.

Lymphocytes from 8 patients and 8 normal control subjects were challenged with 20 lambda of *H. influenzae* antigen at a concentration of 1×10^{10} organisms per milliliter (Fig. 2). All cultures had suppression of uptake upon challenge with this concentration of antigen. The degree of suppression was essentially the same for both the patients' cultures and the normal control cultures. Mean ratio of patient cultures was 0.55 and that for normal controls was 0.56.

Lymphocytes from 5 patients and 5 control subjects were challenged with 20 lambda of *H. influenzae* antigen having a concentration of 3×10^7 organisms per milliliter. There was mild stimulation with no essential differences between lymphocytes of patients and normal control subjects (Fig. 3). Mean ratio of patient cultures was 1.16, while that for the normal controls was 1.05.

Challenging lymphocytes of 10 control subjects and 12 patients with 20 lambda of mixed bacterial antigen resulted in a moderate stimulation of the majority of cultures of both patients and normal control subjects. The difference between the two groups is not significant (Fig. 4), with mean ratios of 3.7 for patient cultures and 4.4 for normal controls.

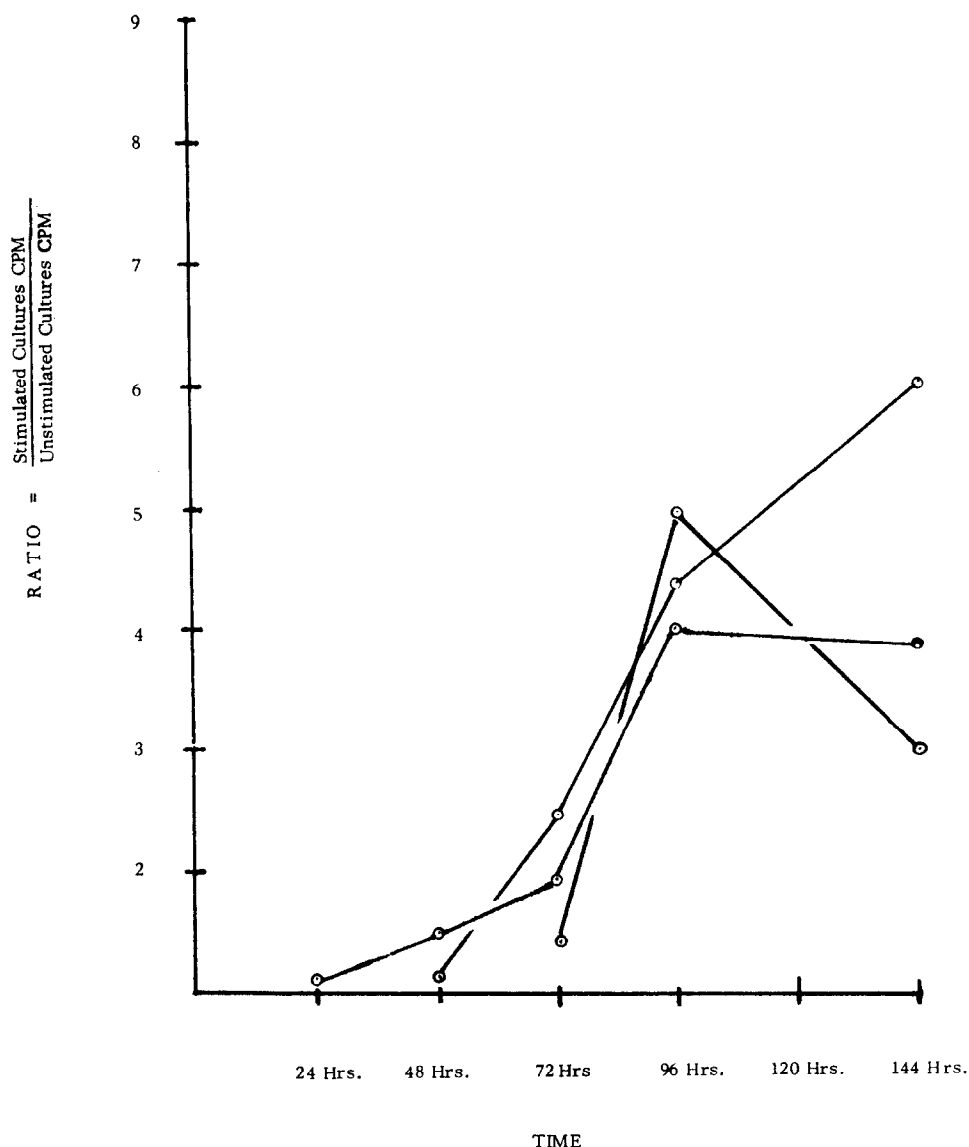


FIG. 1. Time-response curves, normal control subjects challenged with mixed bacterial antigen and cultured for 24 hours, 48 hours, 72 hours, 96 hours, and 144 hours.

Runs were performed with lymphocytes from 4 patients on steroids with the addition of 20 per cent of the patient's own plasma to the culture medium. Lymphocytes from 5 normal control subjects were also run in the same manner (with addition of 20 per cent of their own plasma to the culture medium) for comparison (Table I). All lymphocytes from patients on steroids had *reduction* of ^3H -thymidine uptake when cultured with the addition of their own plasma to the culture medium, whereas those from 4 of the 5 normal controls had a mild *increase* in ^3H -thymidine uptake with the addition of autogenous plasma. Serum

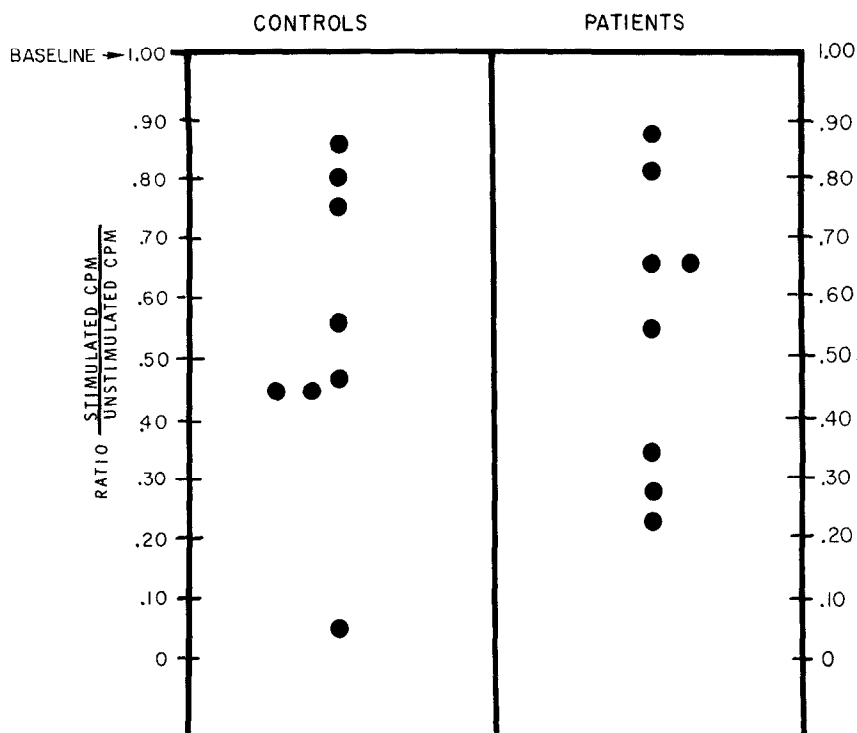


FIG. 2. Ratio (stimulated cultures (c.p.m.)/unstimulated cultures (c.p.m.)) of lymphocyte responses upon challenge with 20 lambda of *H. influenzae* antigen at a concentration of 1×10^{10} organisms per ml.

from one patient on steroids was added to the culture medium of lymphocytes from 2 normal control subjects. This also resulted in a drop in thymidine uptake (Table I).

DISCUSSION

The results of our studies appear to indicate that there is no difference between normal individuals and intrinsic asthmatic patients in the response of lymphocytes to challenge with either mixed bacterial or *H. influenzae* antigen. The mixed bacterial antigen seems to be moderately stimulatory for the cells of both the intrinsic asthmatic and of the normal control persons. The degree of response is the same for the intrinsic asthmatic patients as it is for the normal control subjects (Fig. 4).

It is not known as yet which fractions of the mixed bacterial vaccine are responsible for the lymphocyte stimulation. Preliminary work at our laboratory suggests that fractions from beta hemolytic streptococci may be responsible for most of the stimulation.

As some of the patients in this study were under treatment with steroids which have previously been shown to inhibit human leukocyte mitosis,^{14-16, 22} the response of the lymphocytes upon challenge with mixed bacterial antigens was compared with the response of those from other groups (Fig. 5). The degree of

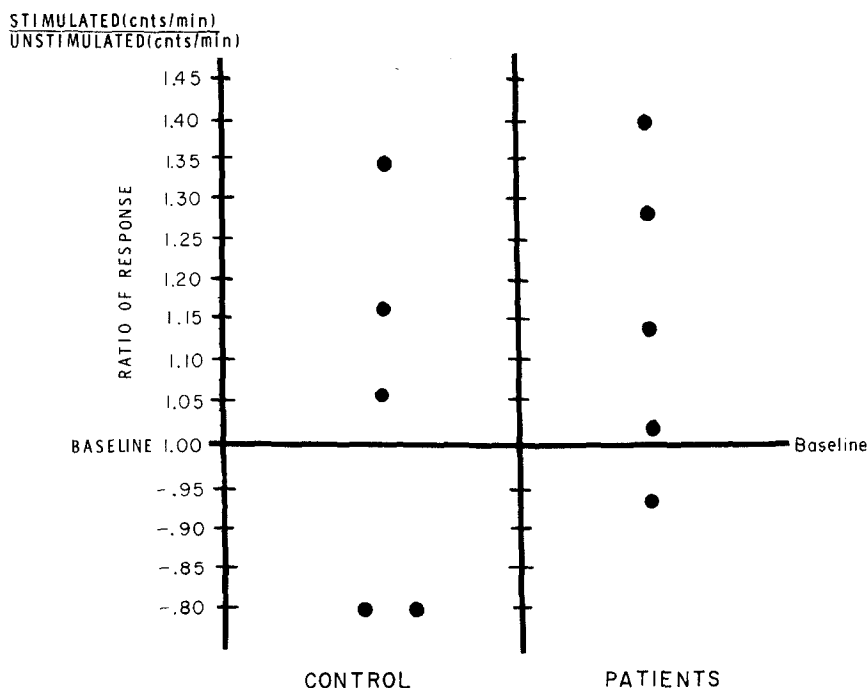


FIG. 3. Ratio (stimulated cultures (c.p.m.)/unstimulated cultures (c.p.m.)) of lymphocyte responses upon challenge with 20 lambda of *H. influenzae* antigen at a concentration of 3×10^7 organisms per milliliter.

response of lymphocytes from intrinsic asthmatic patients on steroids appears to be somewhat lower than the mean of the response of lymphocytes from patients not on steroids and also those from the normal control subjects. The mean ratio for patients on steroids is 2.6 and that for normal controls is 4.4. This difference is not highly significant ($p > 0.05$).

The responses of the lymphocytes from both the normal control subjects and the intrinsic asthmatic patients were also the same upon challenge with *H. influenzae* (Figs. 2 and 3). The data would seem to indicate that the lymphocyte response to this material is related to antigen concentration, with mild stimulation at lower concentrations and suppression at high concentrations. Besides being typical of the dose response to antigens, this observation also is reminiscent of the lymphocyte response to endotoxin, as reported by both Oppenheim and Perry¹⁵ and Heilman.¹⁶ Whether in fact the lymphocyte response to *H. influenzae* seen in this study was due to endotoxin awaits further study.

While the responses of lymphocytes from both intrinsic asthmatic patients and from normal control subjects appear to be essentially the same upon challenge with the various bacterial antigens, a difference between the two groups was noted in the course of the study. When the counts per minute of unstimulated lymphocytes from the patients were compared with unstimulated cells from the normal control subjects, there appeared to be an increased base-line rate of ^3H -thymidine incorporation by the lymphocytes from some of the intrinsic asthmatic patients. Unstimulated lymphocytes from the 11 normal individuals

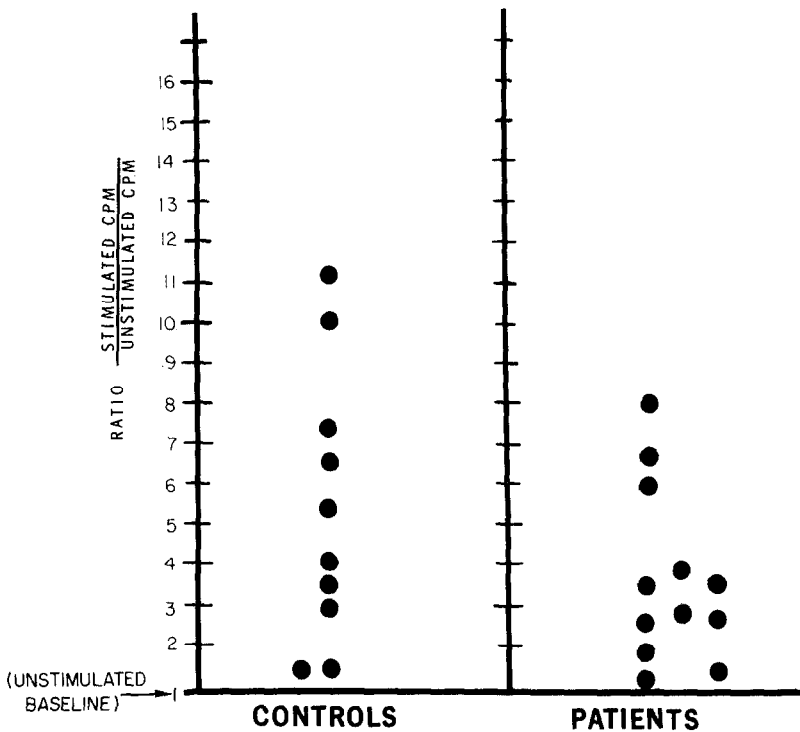


FIG. 4. Ratio (stimulated cultures (c.p.m.)/unstimulated cultures (c.p.m.)) of lymphocyte responses upon challenge with 20 lambda of mixed bacterial antigen.

gave base-line readings which ranged from 300 c.p.m. up to 4,500 c.p.m., with a mean value of 2,500 c.p.m. Unstimulated lymphocytes from the 16 intrinsic asthmatic patients gave base-line readings ranging from 1,625 c.p.m. to 14,500 c.p.m., with a mean of 6,300 c.p.m. (Fig. 6). This difference is statistically significant ($p < 0.05$). The reason for this apparent increase in base-line activity of lymphocytes from some of the intrinsic asthmatic patients is not known at present. The specimens in this study represent sampling at one point in time from patients with fluctuating disease patterns. Further investigation is needed to determine whether the unstimulated activity of the lymphocytes changes with time or fluctuates with the disease pattern. Also, in this study, all lymphocytes were cultured in medium containing 10 per cent fetal calf serum. Whether the lymphocytes from some of the intrinsic asthmatic patients are responding to some fraction of the fetal calf serum which does not affect normal lymphocytes also needs to be determined.

It is possible that a chronic subclinical infection may be responsible for continuous stimulation of lymphocyte transformation, thus producing an increase in base-line activity, but it would seem that such a prolonged stimulation should result in either tolerance or in hypersensitivity to the appropriate antigens.^{10, 11, 17-19} Results of this study appear to indicate that there is neither tolerance nor hypersensitivity of the lymphocytes from intrinsic asthmatic patients when compared to the responses of normal subjects upon challenge with

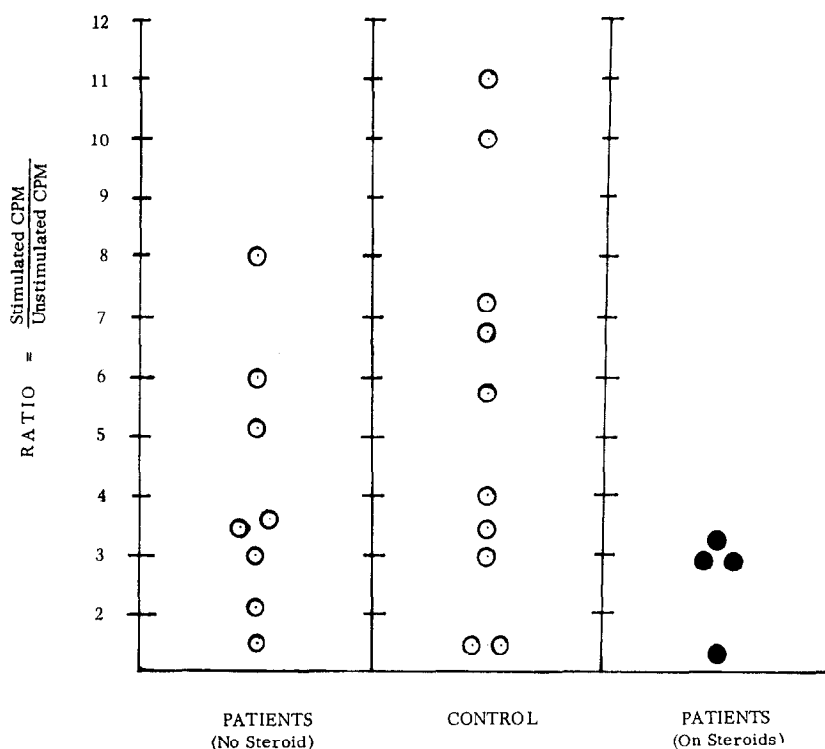


FIG. 5. Response ratios of lymphocytes from 4 patients on steroids (**solid circles**) versus those from 8 patients not on steroids and also 10 normal control lymphocytes upon challenge with mixed bacterial antigen. Mean response ratio for patients on steroids = 2.6; mean response ratio for patients not on steroids = 4.1; mean response ratio for control subjects = 4.4.

the bacterial antigens used in this study. Challenges with viral agents still need to be done.

It is of interest that 4 of the 5 patients on steroids had lymphocytes with a base-line activity about 5,000 c.p.m. These lymphocytes demonstrated a fall in base-line activity upon addition of 20 per cent autogenous serum to the culture medium. When lymphocytes from 2 normal control subjects were incubated in culture medium to which was added 20 per cent serum from a patient on steroids, there was also a drop in base-line activity (Table I). Presumably this fall was due to the therapeutic steroid these patients were receiving at the time of the study, since lymphocytes from normal control subjects and also lymphocytes from patients not on steroids did not show this fall of base-line activity in the presence of autogenous serum.

Finally, both Hall and co-workers²⁰ and Warwick and Haslam²¹ have raised the possibility that intrinsic asthmatic patients may be experiencing an autoimmune response to their own tissue. The data for this are not yet completely convincing. However, if this is so, one might expect to see elevated base-line activity of lymphocytes from some intrinsic asthmatic patients. This possibility is presently under investigation in our laboratory.

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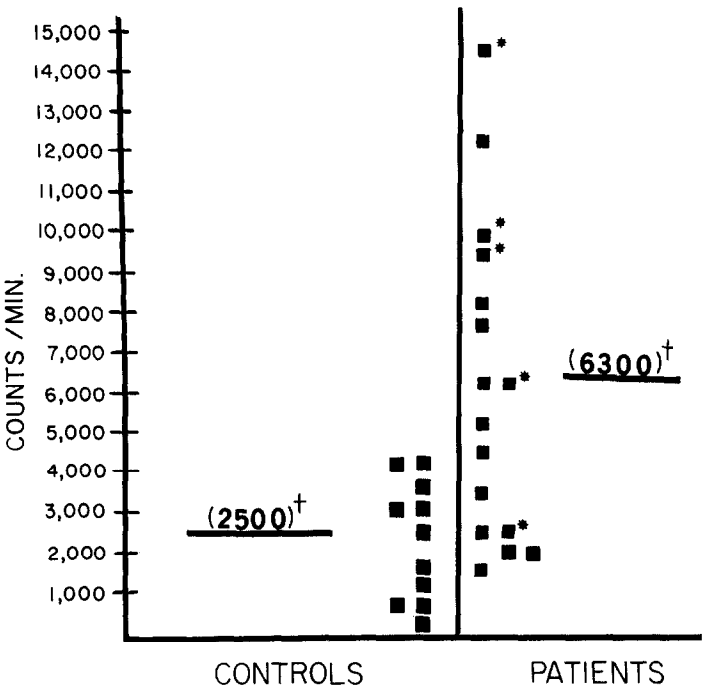


FIG. 6. Unstimulated lymphocyte cultures expressed as counts per minute, patients versus normal control subjects. *Patients on prednisone. †Mean value.

TABLE 1. Comparison of ³H-thymidine uptake in patients with intrinsic asthma with and without steroid therapy

	Base line (c.p.m.)	Base line + 20 % autogenous serum (c.p.m.)	Ratio = $\frac{\text{base line + 20 \% serum}}{\text{base line}}$
Patients on steroids			
T. H.	10,428	5,409	0.51
R. M.	6,381	4,115	0.64
J. R.	9,533	5,032	0.53
J. B.	2,253	1,757	0.77
Patients not on steroids			
N. F.	4,426	5,569	1.25
S. M.	2,270	2,163	0.95
E. T.	7,944	9,411	1.18
Control subjects (culture with own serum)			
T. C.	4,568	5,243	1.14
W. K.	1,963	1,706	0.86
W. A.	3,460	5,971	1.72
R. D.	4,261	4,550	1.06
J. F.	3,903	5,908	1.51
		Base line + 20 % of patient serum (T. H., on steroid)	
Control subjects (culture with patient's serum)			
T. C.	4,567	1,673	0.37
G. S.	2,891	2,248	0.77

The addition of autogenous serum appears to suppress mitotic activity, probably because of its content of steroids. Autogenous serum has no influence in patients off steroids. Suppression occurs in control subjects if the serum of steroid-treated patients is added.

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