IMMUNOLOGICAL INVESTIGATIONS, 15(1), 61-71 (1986)

PROLIFERATIVE RESPONSE IN SOLID CULTURE OF T CELLS FROM PATIENTS WITH EXTRINSIC BRONCHIAL ASTHMA

J. Carvajal, A. Rivas, D.P. Ponce and N.E. Bianco
The Clinical Immunology National Center. The Pathology Institute and the World Health Organization Collaborative Center in Clinical Immunology, WHO/PAHO-SAS/UCV, Apartado 50647, Correos de Sabana Grande, Caracas 1051, Venezuela.

ABSTRACT

T cells proliferative response in both liquid and solid culture conditions (the later prevents direct cell to cell contact) was investigated in nineteen Extrinsic Bronchial Asthma patients (EBA). Tetradecanoyl-phorbol-13-acetate (TPA), a T cell mitogen was used as mitogenic stimulus in both liquid and solid conditions. While the response to phytohemaglutinin (used in liquid culture as reference) was intact, the EBA group showed a significant reduction in the proliferative response to TPA in both culture conditions.
Furthermore, when adherent cells were removed prior to TPA stimulation, while the control group showed a significant decrease in proliferation, the already depressed low proliferation of the EBA group remained unchanged. These results suggest the prevalence of suppressor signals in EBA and may indicate heterogeneity of the suppressor cells pool since high levels of total serum IgE were also present in the same patients.

INTRODUCTION

The possible relationship between altered cell mediated immunity (CMI) and the immunopathology of Extrinsic Bronchial Asthma (EBA) have received mayor research attention in recent years (1,2,3). Furthermore, immunoregulatory defects related to IgE synthesis have been also implicated (4,5,6).

In the present investigation, we have examined the behavior of T cells from patients with EBA in solid culture conditions

0882-0139/86/1501-0061$3.50/0

which prevents direct cell to cell contact allowing to study the susceptibility of the cells to the influence of regulatory signals.

MATERIAL AND METHOD

Patient Population

Nineteen patients with the diagnosis of Extrinsic Asthma (EBA) ranging from 8 to 30 years were studied. In sixteen of them, allergic rhinitis was also present. Eight out of the 19 patients were under topic nasal treatment with Sodium Cromoglicate (4% solution); none of the 19 patients were receiving any systemic medications including steroids. Twenty healthy non-atopic volunteers (age range: 17 to 30 years) of both sexes were included as control group.

Allergy Laboratory Work-up

The patient population were subjected to allergy work-up performed in our center as previously reported by Ponce et al. (7). Briefly, routine blood counts, stool for ova and parasites as well as total serum IgE (Prist) and RAST test to: D.pteronisynnus, D.pharinnae, Ragweed and Mix molds (Pharmacia, Upsala, Sweden and Hollister Stier Laboratories, Spokane, Wa.) were performed.

Isolation of Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood (10 UL/ml) diluted 1:2 with phosphate buffered saline (PBS) pH 7.4 by centrifugation on ficoll-hypaque density gradients (8). PBMC were washed three times with PBS and suspended in the appropriate culture mediun. Cell viability was greater than 98% by trypan blue dye exclusion.

Depletion of Adherent Cells

Adherent cells were depleted by incubation of $3x10^7$ PBMC in 10ml of RPMI 1640 supplemented with 25mM hepes buffer, 2ml L-glutamine, 100 U/ml of penicillin, 100 ug/ml streptomicin (Grand Island Biological Co.) and 10% decomplemented pooled human serum (referred as complete medium) on plastic petri dishes 15x100mm (Lab. Tek Products, Wasperville, IL1) for 90 min at 37°C. The non-adherent cells were recovered by gently washing with warm RPMI 1640 medium containing 10% human serum. The cells were washed two times with PBS and suspended in appropriate culture medium. The non-adherent cells fraction contained over 94% T cells (E^+) and 2-3% of monocytes searched by Giemsa stain.

Lymphocyte Stimulation in Liquid Culture

PBMC ($1x10^5$) were cultured in complete medium and stimulated with 15 ug/ml of PHA (Welcome Bourrougs, England) or 10^{-6} M tetradecanoyl-phorbol-13-acetate (TPA, Consolidated Midlan Co. N.Y.). TPA was dissolved in dimethyl sulphoxide (DMSO) at 10^{-2} M and subsequently diluted to working concentration in RPMI 1640. The selected DMSO concentration has been shown not to alter ^{3}H-thymidine incorporation. The stimulation was carried out in round bottom microtiters plates (Limbro Division Flow Lab. Handen Co.) in a humified atmosphere of 5% CO2 air mixture for 72 hours. 18 hours before the end of incubation period, the cultures were pulsed with 1uCi/well of ^{3}H-thymidine (New England, Boston, Mass); the labelled DNA was harvested onto glass-fiber filter in a MASH II apparatus. The thymidine incorporation was determined in a liquid scintillation counter. The degree of stimulation was expressed as Δ cpm calculated as mean cpm for each mitogen concentration minus mean cpm of stimulated culture.

Lymphocyte Stimulation in Solid Culture

Lymphocyte stimulation in solid culture was performed according to Kondracki and Milgron (9), as previously standarized in our laboratory (10,11). Briefly, 2% (w/v) agarose (Marine Colloids, Springfield, N.J.) in destilled water was mixed with equal volume 2X-RPMI 1640 supplemented with 200 U/ml penicillin and 200 ug/ml streptomycin (Gibco Grand Island, N.J.). PBMC or non-adherent cells were adjusted to a concentration of $8x10^6$ cells/ml in RPMI 1640; then 50ul of cell suspension were transferred to 10x75mm glass tubes (Fisher Scientific, Pa.) containing 50ul of 1% agarose in RPMI 1640. At this step, the tubes contained a suspension of non-aggregated lymphocytes ($4x10^5$ cells/tube in 100ul of 0.5% agarose solid medium). The lymphocyte suspension was then overlaid with 100ul of RPMI 1640 containing the desired dose of mitogen and 20% of heat-inactivated human pooled serum. Triplicate cultures were incubated at 37°C in 5% CO2, 95% humidity chamber for 72 hours. In the present study, TPA was utilized at predetermined optimal dose of 10^{-6} M. 6 hours prior to culture end, 0.4 uCi of H-thymidine (1 uCi/mM; New England Nuclear Boston, Mass) in 10ul of RPMI 1640 were added to each glass tubes. Cultures were terminated by placing them a -20°C; if no processed immediately, they were stored for up to 1 month. Cultures were then processed by a modification of the method described by Peters (9,12). Data was expressed in count per minute (cpm).

Statistical Analysis

Comparison between groups were based on student T test.

RESULTS

Pertinent Immunodiagnostic Data

The total serum IgE geometric mean level for the EBA group was 939 IU (nine had values < 1000 I.U. and 10 between

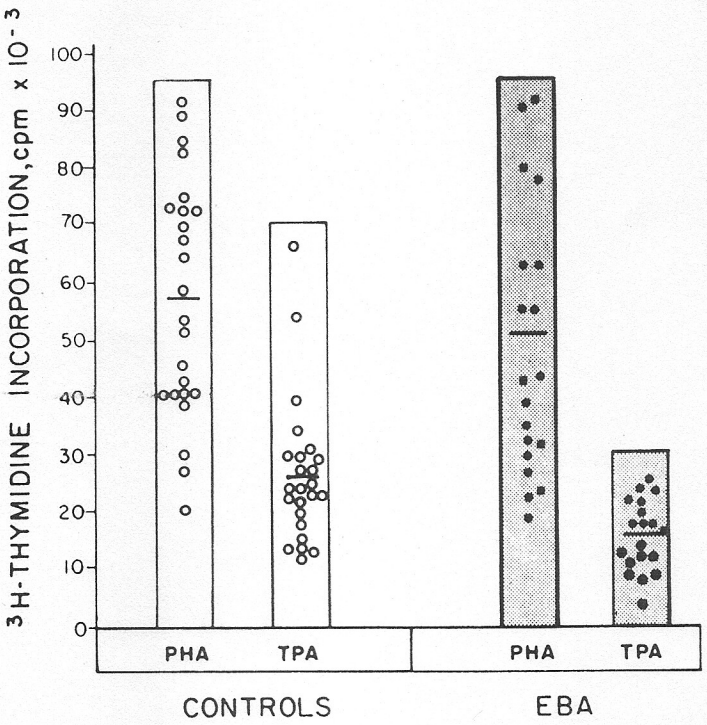

FIGURE 1

^{3}H-thymidine incorporation by PBMC stimulated by PHA and TPA in liquid culture conditions. PBMC controls donors (o); extrinsic bronchial asthma patients (EBA) (•). The horizontal lines indicates their mean respectively. Each point represents the mean of triplicate cultures.

1000-5000) while for the control group was 56 IU (range 15-150) being the difference statiscally significant ($p<0.001$). The EBA patients were most frequently sensitized to: D. pteronyssinus (82%), D.pharinnae (78%), house dust (58%) and ragweed (17%). In neither patients or controls other IgE levels influencing factors were detected including helminths.

Mitogen Response to PHA and TPA in Liquid Culture

The proliferative response to PHA and TPA in 19 EBA patients was examined; as shown in Figure 1, the response

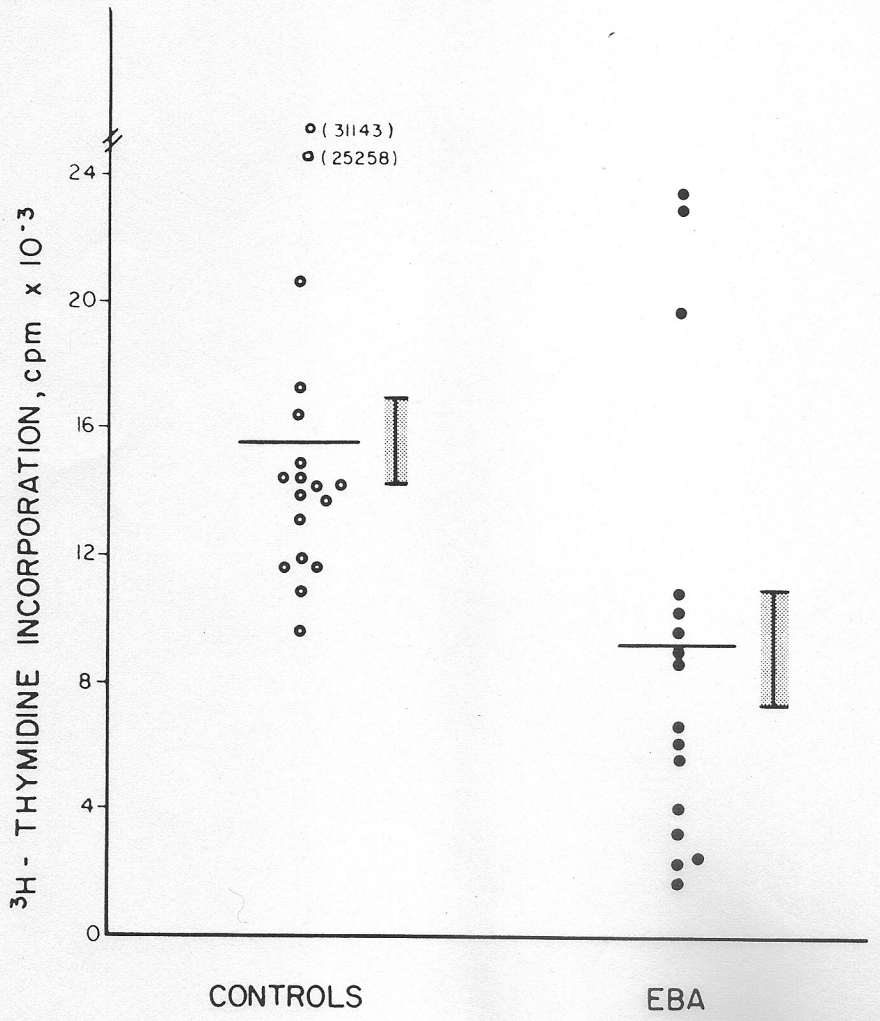

FIGURE 2

$4x10^5$ PBMC/tube from controls (o) or EBA patients (•) were stimulated with TPA (10^{-6} M) for 48 hours in solid culture. Line bars indicate mean + s.e.

to PHA in patients with EBA was comparable to values obtained from healthy controls (54.315 ± 54.68 cpm, mean ± s.e.), while the response to TPA was lower (15.862 ± 1.287 vs 25.857 ± 2.600 cpm) than that obtained in the control group ($p < 0.001$); spontaneous proliferation were 247 ± 28 cpm for the EBA group and 249 ± 23 for the control group.

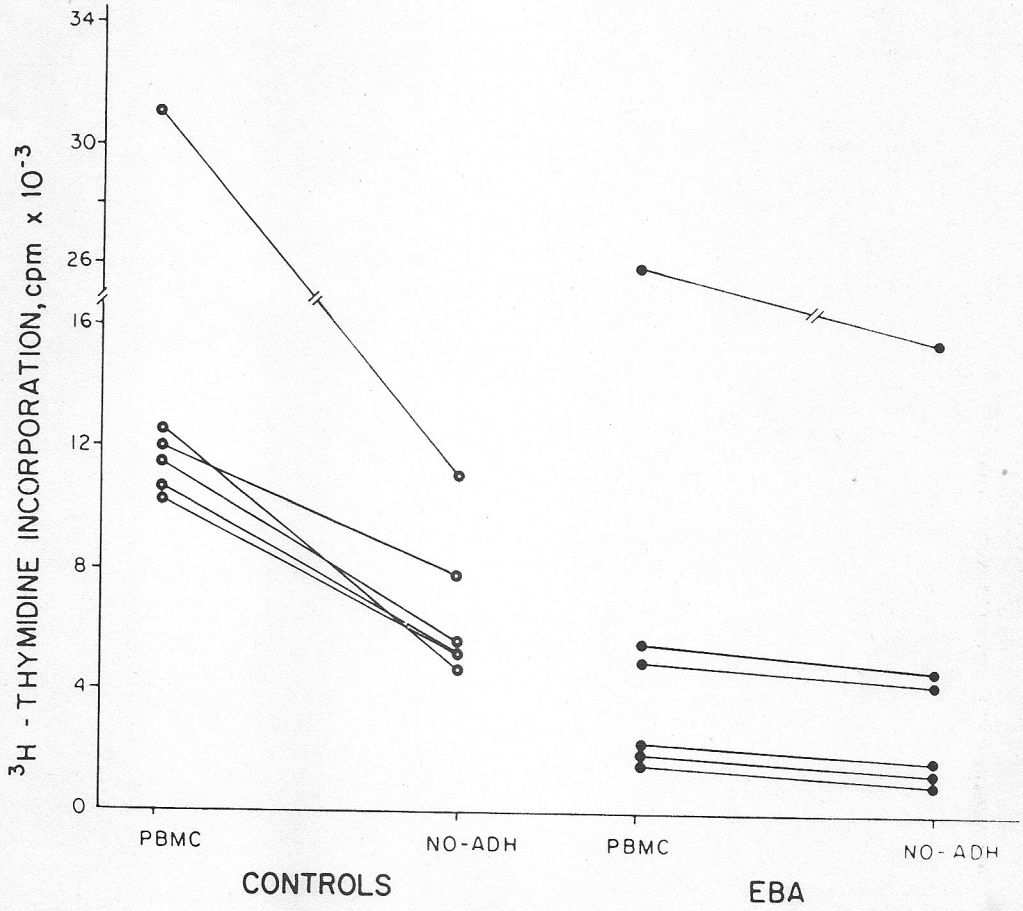

FIGURE 3

Effect of remotal of adherent cells on TPA proliferation in solid culture. PBMC on no-ADH from controls (o) on EBA patients (●) were incubated with TPA (10^{-6} M) in solid culture. Each point represents the mean of triplicate cultures.

Mitogen Response to TPA in Solid Culture

We examined the PBMC proliferation capacity in solid culture in the presence of TPA; in 16 out of 19 EBA patients, the response was significantly lower (9.268 $\pm$ 1.818 cpm) than those from the control group (15.410 $\pm$ 315 cpm) ($p < 0.001$). (Figure 2).

Influence of Removal of Adherent Cells

Under solid culture conditions and in the presence of TPA, experiments with removal of adherent cells were carried out in six EBA patients and controls. While there was a marked and significant reduction in the proliferative responses in the control group, little non-significant modificantion was observed in the EBA group (Figure 3). Spontaneous proliferation of both EBA and controls non-adherent population was similar.

DISCUSSION

In recent years the probable mechanism underlying the immunopathology of EBA have been linked to defects in cell mediated immunity (CMI), particularly to abnormalities in the immunoregulatory process, which controls IgE synthesis. The data accumulated thus far, is conflicting with remarkable variability from the several reports (6,14,15,16), offering no concrete evidences to support CMI or regulatory disturbance in patients with atopic diseases.

Furthermore, Armistead et al. (17) showed in nine patients bearers of eczema, EBA or rhinitis associated with very high levels of serum IgE, comparable results with the control group, when T cells suppressor activity was assesed. The T4/T8 compartment were intact as well the response of Con-A or histamine generated T suppressor cells, when explored in vitro to inhibit proliferation to mitogens or specific antigens which included house dust mite and D. pteronyssinus.

We have approached the study of regulatory T cell function in patients with EBA, using solid culture conditions previously standarized in our laboratory (10,11).

This in vitro assay prevents direct cell to cell contact, allowing to investigate active cell communications and responses to various kinds of signals (9,13). As reference,

proliferative responses to PHA in liquid culture was also explored. Patients selection was uniform; the majority of them, showed the combined diagnosis of EBA and atopic rhinitis and serum IgE levels ranged from 250-5000 IU, which differs significantly from normal values stablished in our center by Ponce et al. (7). Furthermore, the patient population was mainly sensitized to D.pteronyssinus, D.pharinnae, house dust and to ragweed (17%), confirming previous report from our center by Ponce et al. (7).

12-O-tetradecanoylforbol-13-acetate (TPA), was selected for stimulation in solid culture conditions. Our experiments showed, that in standard liquid culture conditions, while the response to PHA was comparable in both EBA and control groups, the patient population proliferative capacity to TPA was reduced as expressed by the significative difference of their respective means. Three of the controls had high thymidine incorporation while one of the EBA patient was slighthy low. These latter findings are probably related to individual responses as often seen in in vitro cell cultures.

When the response to TPA was investigated in solid culture condition, 13 out of 16 showed a decrease in proliferating capacity, being the difference between both groups statistically significant. Again, we noted that three EBA patient showed high thymidine incorporation, reflecting the individual variability mentioned above. It is also important to note that no relationship was found between total IgE levels and T cells proliferative responses to TPA in the patients group. At this point, is important to stress, that since in solid culture conditions, direct cell to cell contact is prevented, the proliferative response may originate from direct TPA-cell interaction, from the interaction TPA-accesory cells or from both (11,13). Furthermore, since TPA is a T cell mitogen (18,19), the reduced proliferative response to TPA observed in EBA patients, suggested the presence of suppressor signals influencing the proliferation capability of some of the T cells pool. When adherent cells were removed prior to

stimulation with TPA, a significant decrease in proliferative response in the control group was observed while the already reduced response in the EBA group remained unchanged. These results could indicate that suppressor signals may be prevalent in the in vitro cell responses of patients with EBA. Since EBA is characteristically associated with high IgE levels, the possibility of a heterogeneous abnormally functioning suppressor cells pool, operating at different levels in atopic patients should be further investigated.

ACKNOWLEDGMENT

This work was supported by grants from Ministerio de Sanidad y Asistencia Social and CONICIT (Fortalecimiento de Centros. We thank Amanda González for secretarial assistance.

REFERENCES

1. Harper, T.B., Gaumer, H.R., Waring, W., Brannon, R.B and Salvagio. J.E. Clin Allergy 10, 555-563 (1980).

2. Wang, S.S.M., McGeady, S.J. and Mausmann, Jr.H.C. Clin Allergy 13, pp.323-328 (1983).

3. Strannegard, I.L. Int Arch Allergy Appl Imm 58, pp. 175 181 (1979).

4. Saxon, A., Morrow, C., Stevens, R.H. J Clin Invest 65 pp. 1457-1468 (1980).

5. Ishizaka, K. Adv Immunol 23, pp. 1-52 (1976)

6. Martínez, J.D., Santos, J., Stechschulte, D.J. and Abdo N.I. J Allergy Clin Immun 6, pp. 485-490 (1979).

7. Ponce, D.P., Anderson, O., Ilja, R., Monzón, A. and Bian N.E. Clin Allergy 13, pp. 521-528 (1983).

8. Boyum, A. Scand J Clin Lab Invest Suppl 21, 97 pp. (1968).

9. Kondracki, E. and Milgrom, F. J of Immunol 118: pp. 3 387 (1979).

10. DeFreitas, M.T., Kondracki, E., Pérez-Rojas, G. and N Bianco. Immunol. Commun 11, pp.243-251 (1982).

11. Rivas, A.J., Machado, I., Kondracki, E. and Bianco, N.E. Gerontology 31,pp. 39-45 (1985).

12. Peters, J.H. Exp Cell Res 74, pp. 179-186 (1972).

13. Kondracki, E. Int Arch Allergy Appl Immun 61, pp. 125-135 (1980).

14. Valverde, E., Vich, J.M., Huguet, J., García-Calderón, J.V. and García-Calderón, P.A. Clin Allergy 13, pp. 81-88 (1983).

15. Grove, D.I., Burston, T.O., Welby, M.L., Ford, R.M. and Forbes, I.J. J of Allergy and Clinical Immun 55, pp. 152-156 (1975).

16. Leung, D.Y.N., Sargan, J.A., Frankel, R., Lareau, M. and Geha, R.S. J Clin Invest 72, pp. 1482-1486 (1983).

17. Armistead, J.G. and Ewan, P.W. J Clin Lab Immun 12, pp. 179-185 (1983).

18. Touraine, J., Hodden, J., Touraine, F., Hodden, E., Estensen, R. and Good, R. J Exp Med 145: pp. 460-465 (1977).

19. Sugawara, I. and Ishizaka, S. Clin Immun Immunopathol 26, pp. 299-308 (1983).