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Dependence of Proliferative Activity of Lymphocytes on Basal Levels of Lymphocytic Cyclic Nucleotides in Lepromatous Leprosy Patients[1]

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Lepromatous leprosy patients are characterized by a significant impairment of their immune responses, which is expressed through the depression of delayed-type hypersensitivity (DTH) reactions to mycobacterial antigens ([6, 13–15, 17, 22]). The mechanism of those disturbances has so far been little studied. Since lymphocytes play a leading role in reducing DTH responses, it seems important to study the state of the lymphocytic regulatory system at the molecular level, the system of cyclic nucleotides being the most important in this respect.

Cyclic nucleotides, namely, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), in humans and in highly organized animals are known to be secondary mediators between an external stimulant and the cell response to it. The neuroendocrine system serves as a primary mediator ([10]). The development of an immune response is inhibited by high basal levels of cAMP, and is stimulated by cGMP in immune competent cells ([10–12]). The dependence of functional activity of lymphocytes on the levels of their cyclic nucleotides has not been studied in leprosy. It was the aim of our investigation.

MATERIALS AND METHODS

Thirty patients with lepromatous leprosy (LL, by Ridley-Jopling) 31–58 years old were studied; 11 females and 19 males. Eighteen patients had clinical manifestations of an active leprosy process in the form of diffuse and/or localized infiltrates and/or lepromas in skin. Histological investigation showed residual granulomas of the lepromatous type containing moderate numbers of solid or degenerating mycobacteria. Twelve patients had no clinical or histological signs of an active process. None of the patients had endocrine pathology and, hence, none were being treated with hormones. At the time of the investigation, all of the patients were being treated with sulfones, mostly in combination with rifampin or prothionamide. Fourteen healthy volunteers, 32–57 years old, served as controls; four were tested twice (Table 1). They took no medication the day before our investigation.

Lymphocyte isolation from peripheral blood. Lymphocytes were isolated according to the method described elsewhere ([3]) with a slight modification. Aliquots of blood (2 ml) taken from the ulnar vein were distributed into heparinized glass tubes, diluted 1:1 with phosphate buffer (pH 7.2), and layered on Ficoll-Isopaque with a density of 1.077 (Pharmacia Fine Chemicals, Sweden). The tubes were centrifuged at 400 × *g* at 18–20°C for 40 min. The yield of mononuclear cells was 44–75% from the whole blood. The isolated cell pool contained 80–84% lymphocytes, 15–17% monocytes, and 1–3% granulocytes. The cells were washed twice with phosphate buffer, and counted three times using Goryaev's chamber.

Extraction of cyclic nucleotides from lymphocytes. Two-tenths ml of lymphocyte suspension with a strictly determined number of cells was pipetted into a glass tissue grinder (Gallenkamp, U.K.) which was placed into liquid nitrogen. Homogenization was carried out by one and the same worker and ended when 82–85% of the cells were destroyed. Homogenates were washed three times with cold ethanol. Ethanol-denatured proteins were sedimented by means of centrifugation. The supernatants were placed into a drying apparatus with partial vacuum (made in our laboratory). Dried extracts were

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dissolved in Tris-EDTA buffer (pH 7.5), frozen, and stored at −50°C. The inter-test difference in the extraction of cyclic nucleotides from lymphocytes seemed to be due to various contaminations of nonlymphocytic cells (variation ≤ 4%) and to different amounts of disrupted cells (variation ≤ 3%). The total error did not seem to exceed 7%.

Estimation of cyclic nucleotides. Cyclic nucleotides were estimated according to Tovey, *et al.* ([25]); Steiner, *et al.* ([23]), and Albano, *et al.* ([1]), using "Amersham" kits. For cGMP samples, a liquid scintillant mixture was prepared on the basis of toluene/triton X-100, according to Patterson and Greene ([20]); for cAMP samples, on dioxane according to Bray ([4]). Radiometry of the samples was carried out in a liquid scintillation counter SBS-2 (U.S.S.R.). The concentrations of cyclic nucleotides were expressed as pmoles/10^6 cells.

Lymphocyte blast transformation test (LTT). LTT was carried out according to the method described elsewhere ([18]) with a slight modification. In 2 ml of whole blood ("Serva" heparin was used as anticoagulant in a dose of 30 units/ml), the absolute leukocyte number was counted using Goryaev's chamber (U.S.S.R.). The relative lymphocyte number was estimated in azur-eosin-stained smears. Phytohemagglutinin-M (PHA; Calbiochem) in a dose of 0.05 mg/ml and purified protein derivative (PPD; Leningrad Institute of Vaccines and Sera, U.S.S.R.) in a dose of 0.02 mg/ml served as stimulators. Five × 10^5 leukocytes were cultivated for LTT with PHA and 10^6 cells for LTT with PPD. The cells were incubated in 10 ml flat-bottom, tightly capped glass flasks at 37°C in 3 ml of medium-199 (U.S.S.R.) supplemented with 10% homologous serum pool from healthy donors of the AB blood group, 200 units/ml of benzyl penicillin sodium and 100 μg/ml of streptomycin sulfate. The cells for LTT with PHA were incubated for 96 hr; for LTT with PPD, for 120 hr. All of the cultures, whether stimulated or not, were set up in triplicate. Two μCi of ^{3}H-thymidine ("Isotope," specific activity 2.0–2.5 Ci/mMole; U.S.S.R.) was pipetted into each culture 24 hr before the end of incubation. After incubation, the cultures were washed twice with 3% acetic acid and once with 5% trichloroacetic acid (TCA). The cellular sediment was hydrolyzed in 0.5 ml of 5% TCA at 90°C for 30 min to isolate DNA. The resulting hydrolysate was transferred into counting flasks with 10 ml of the liquid scintillant mixture ([4]). Radiometry was carried out in the liquid scintillation counter SBS-2.

The final result was expressed as the difference in radioactivity between the test cultures and the control cultures. For statistical analysis, the results obtained were transformed into counts per minute (cpm) by 10^4 lymphocytes stimulated with PHA. In the case of stimulation with PPD, we used counts per 10 min by 2 × 10^5 lymphocytes because of the low incorporation of ^{3}H-thymidine. According to the laws of nuclear statistics ([26]), the more the count, the less the possibility of error. Statistical variations between triplicates averaged 11% for LTT with PHA, 20% for LTT with PPD, and 15% for control unstimulated cultures.

RESULTS AND DISCUSSION

When discussing and interpreting the results obtained, we decided not to consider the levels of cyclic nucleotides and LTT intensity taken separately, because these questions in relation to leprosy have been extensively studied ([2, 6, 13–15, 17, 21, 22]). There seems to be doubt about some data obtained with the LTT test.

In some leprosy patients we did not observe a stimulation but, rather, a depression of lymphocytic proliferation when stimulated with PPD. In such cases, after subtracting the counts of the control cultures from the counts of the test cultures, we obtained a negative value (Table 2). The following facts might be indicative of the immunologic nature of this "phenomenon": a) The "phenomenon" was observed in leprosy patients but not in healthy persons. b) It was observed when stimulating with PPD but not with PHA. A similar depression of the proliferative response of leprosy lymphocytes to tuberculin was also noted by other authors ([7, 9]). c) No technical errors were possible since the process of preparing the test and control cultures was standardized, and the degree of scintillation checked by the method of internal standards ([26]) proved to be similar in both test and control cultures. While performing rank correlation

TABLE 1. *Basal levels of cyclic nucleotides[a] and intensity of LTT[b] in healthy persons.*

Cyclic nucleotides		LTT with	
cAMP	cGMP	PHA	PPD
[c] 11.06	—	2,878	2,840
[c] 8.96	—	4,386	20,880
[c] 16.07	—	3,866	—
[c] 11.23	—	5,046	129,440
[c] 28.46	—	2,164	10,180
[c] 20.76	—	4,376	2,660
[c] 14.86	—	2,076	17,500
[c] 10.00	—	7,244	43,100
11.33	0.467	4,520	8,180
6.72	0.080	6,066	9,280
16.98	0.025	9,246	17,040
9.75	0.468	30,118	107,740
7.35	0.612	10,330	259,600
7.10	0.114	13,678	134,480
2.68	0.235	30,062	241,520
18.82	—	12,312	15,940
25.35	0.371	8,054	21,050
—	0.333	6,266	30,900

[d] ρ: (+0.150), (−0.412), (+0.333), (−0.518)

[a] Levels of cyclic nucleotides are expressed as pmoles/10^6 lymphocytes.

[b] Values of LTT are expressed for cultures with PHA as cpm by 10^4 lymphocytes; for cultures with PPD, as counts per 10 minutes by 2×10^5 lymphocytes.

[c] Data obtained from persons investigated twice.

[d] Arrows bind the columns of the numbers correlated by Speerman's method; ρ (in brackets) = coefficients of such correlation.

TABLE 2. *Basal levels of cyclic nucleotides[a] and intensity of LTT[b] in leprosy patients.*

Cyclic nucleotides		LTT with	
cAMP	cGMP	PHA	PPD
6.14	0.146	—	8,716
28.30	0.200	436	2,440
10.47	0.902	—	1,600
5.01	0.110	9,300	2,887
5.91	0.105	12,328	106,165
6.82	0.181	10,916	335,032
2.39	2.389	—	3,073
13.80	0.450	13,915	−280
11.61	0.170	2,302	—
—	1.010	2,482	6,170
5.23	0.520	5,672	1,072
8.52	0.450	2,884	6,704
13.61	0.396	6,810	—
17.14	6.095	17,966	−1,371
20.29	0.472	3,878	0
33.02	—	2,910	1,740
16.67	—	5,216	27,132
10.80	0.602	15,770	−357
6.36	0.471	—	1,006
8.30	0.262	1,598	—
38.36	0.660	1,866	400
13.30	0.385	6,669	21,975
5.12	0.234	325	—
18.92	—	6,731	230
15.82	2.950	—	3,612
—	0.880	5,384	856
21.40	1.170	—	−368
16.19	1.240	1,326	288
7.24	2.070	760	884
9.90	—	6,476	0

[c] ρ: (−0.065), (−0.091), (−0.480), (−0.415)

[a] Levels of cyclic nucleotides are expressed as pmoles/10^6 lymphocytes.

[b] Values of LTT are expressed for cultures with PHA as cpm by 10^4 lymphocytes; for cultures with PPD, as counts per 10 minutes by 2×10^5 lymphocytes.

[c] Arrows bind the columns of the numbers correlated by Speerman's method; ρ (in brackets) = coefficients of such correlation.

analysis, the negative numbers were also taken into consideration.

The correlations between the basal levels of cyclic nucleotides and the intensity of LTT in healthy persons are given in Table 1. The most marked influence of basal levels of cyclic nucleotides on LTT with PPD was observed, the levels of cAMP being inversely related to LTT intensity ("negative" influence) opposite to cGMP ("positive" influence). A similar but less significant correlation was noted between the LTT intensities with PHA and the levels of cyclic nucleotides. The correlation coefficients were low in this case, but when comparing them by Student's *t* test using Fisher's transformation ([16]), the difference appeared to be significant ($p < 0.05$).

The correlations between the cyclic nucleotide levels in lymphocytes and the LTT intensity in leprosy patients are given in Table 2. The intensity of LTT with PHA stimulation was insignificantly related to cyclic nucleotide levels in the lymphocytes. A

marked negative relationship was observed between the lymphocytic cAMP levels and the values with PPD stimulation ($p < 0.05$). The relationship between the intensity of LTT and the lymphocytic cGMP levels also proved to be significant and negative ($p < 0.025$). The difference in the above correlation coefficient between leprosy patients and healthy persons was statistically significant ($p < 0.001$).

The data obtained indicate that the relationship between the LTT and basal levels of cyclic nucleotides in lymphocytes was not linear (see tables), i.e., the highest level of cAMP does not always correspond to the lowest LTT value, and vice versa. This holds true for the correlation between LTT and the levels of cGMP. Obviously, a cell proliferative program involves a great number of factors, and basal levels of lymphocytic nucleotides, although significant, are only some of these factors, serving as an initial "step," triggering the proliferative program. Further development of this program is mostly dependent on cAMP-mediated, cell protein kinases ([19]). Due to this fact, the correlations investigated could hardly be used with diagnostic purposes for assessment of the immune status of an individual patient, but they may provide insight into certain tendencies in the immunopathology of leprosy.

According to our data and the data of other authors ([8, 11]), cAMP and cGMP are normally reciprocal regulators of cell proliferative programs. Lepromatous leprosy patients seemed to show a significant change in the cGMP role in lymphocytic proliferation. It is interesting that such a change in the cGMP role was noted with PPD stimulation but was absent when PHA was used as the stimulator in LTT. This might be due to a peculiar state of mycobacterial antigen-binding receptors.

Our findings show that the most significant difference between lepromatous patients and healthy persons is that the higher the cGMP levels in their lymphocytes, the weaker the immune response to mycobacterial antigens will be. This seems to be one of the causes of the relatively poor effect of immune stimulators in lepromatous leprosy. Thus, if an immune stimulator is thought to enhance the proliferative activity of lymphocytes by raising the cGMP levels in leprosy, it might exert an "unexpected" depressive effect (summed negative effect of cGMP and cAMP). If an immunostimulator depresses the cAMP levels and increases the cGMP levels, its effect in leprosy does not seem to be distinct (less negative effect of cAMP but more negative effect of cGMP).

These data shed additional light on the problem of the immune defect in leprosy, and should stimulate further research in the immunopathology of the disease.

SUMMARY

The correlation between the lymphocyte blast transformation test and the basal levels of cyclic nucleotides (cAMP and cGMP) in lymphocytes was studied in 30 patients with lepromatous leprosy and 14 healthy persons. Cells in cultures of whole blood were stimulated with PPD and PHA. As compared to healthy persons, leprosy patients showed inversion of the role of cGMP basal levels in proliferative activity of lymphocytes in a response to PPD. The data obtained give rise to a hypothesis which might explain the low effectiveness of immune stimulators in leprosy.

RESUMEN

Se estudió la correlación entre la prueba de la transformación blastoide de los linfocitos y los niveles basales de nucleótidos cíclicos (AMPc y GMPc) en los linfocitos de 30 pacientes con lepra lepromatosa y en 14 individuos sanos. En los cultivos de sangre total las células se estimularon con PPD y con PHA. Comparados con las personas sanas, los pacientes con lepra mostraron una inversión del papel de los niveles basales del GMPc en la actividad proliferativa de los linfocitos estimulados con PPD. Los datos obtenidos dieron lugar a una hipótesis que quizá pueda explicar la baja eficiencia de los estimuladores inmunológicos en la lepra.

RÉSUMÉ

Chez trente malades atteints de lèpre lépromateuse, et chez 14 personnes en bonne santé, on a étudié la corrélation entre l'épreuve de transformation lymphoblastique et les taux de base des nucléotides cycliques (cAMP et cGMP) dans les lymphocytes. Les cellules mises en culture dans le sang total ont été stimulées avec le PPD et la PHA. Lorsqu'on compare les résultats observés chez les malades de la lèpre et chez des individus en bonne santé, on observe chez les premiers

une inversion du rôle des taux de base la cGMP pour l'activité de prolifération des lymphocytes en réponse au PPD. Les données ainsi recueillies fournissent une hypothèse qui pourrait expliquer la faible efficacité des immunostimulants dans la lèpre.

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