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CLINICAL AND IMMUNOBIOLOGICAL EFFECTS OF AN ORALLY ADMINISTERED BACTERIAL EXTRACT*

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Abstract — The effect of a bacterial extract orally administered to 20 children with recurrent infections of the upper respiratory tract, was investigated in a double-blind study. The composition of the peripheral blood mononuclear cells (T and B-lymphocytes, monocytes) and some of their biochemical properties (5′-nucleotidase, β-*N*-acetyl-glucosaminidase and non-specific esterase) were unaffected. In contrast, the allogeneic mixed lymphocyte reaction was significantly increased in patients treated with the bacterial extract. In the treated group the number of infectious episodes decreased significantly and the clinical response correlated positively with the mixed lymphocyte reaction. These findings suggest that the bacterial extract has the capacity of restoring depressed immune functions by acting through the gut-associated lymphoid tissue.

Recurrent infections of the upper respiratory tract are generally associated with defective immune functions. A poor response to pathogenic microorganisms may only reflect a functional disorder, without being the expression of a real immunodeficiency implying serious qualitative and/or quantitative alterations of the lymphohemopoietic system. In these cases antibiotic therapy alone is inadequate while an effective immunopharmacologic approach would remove the major cause of disease. Adoption of bacterial substances as immunomodulators is certainly not new, but the oral administration of a lyophilized bacterial extract (Broncho-Vaxom^{R}) in children with recurrent infections of the respiratory tract presents some interesting aspects. First, the oral route has clear advantages especially in children, second, it might be more effective than other routes considering the important role of the intestinal microflora in maintaining the immune system at a physiologic level of performance.

On the other hand, Broncho-Vaxom has already been shown to be active in a variety of *in vivo* and *in vitro* situations as well as in clinical trials (Puigdollers, Rodés Serna, Hernandez del Rey, Tillo Baruffet & Jofre-Torroella, 1980; Girard & Fleury, 1979; Messerli, Michetti, Sauser-Hall, Stäubli, Taddei, Weiss, Farine & Fux, 1981; Sequeira, 1980).

We reported here the variations of some clinical, immunological and biochemical parameters measured on peripheral blood mononuclear cells (PBMC) of 20 children with recurrent infections of the upper respiratory tract. Beside the clinical observations three major aspects were studied: (a) morphological, by evaluating the various Ig-bearing B-lymphocytes, the different T-cells subsets and the monocyte fraction; (b) biochemical, by determining the activity of membrane-bound 5′-nucleotidase and of lysosomal β-*N*-acetyl-glucosaminidase; (c) functional, by analyzing the patients' alloreactivity in a one-way mixed lymphocyte culture (MLC).

EXPERIMENTAL PROCEDURES

Broncho-Vaxom (BV)

BV is obtained by exposing bacterial microorganisms (see below) to an alkaline lysis. The hydrosuluble part is extracted and lyophilized. One capsule of Broncho-Vaxom *children* (OM Laboratories, Meyrin/Geneva, Switzerland), contains 3.5 mg of the lyophilized bacterial extract of following composition: *Haemophilus influenzae, Diplococcus pneumoniae, Klebsiella pneumoniae* and *ozaenae, Staphylococcus aureus, Streptococcus pyogenes* and

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viridans, Neisseria catarrhalis. The bacterial extract is mainly composed of ribonucleoproteins (OM Laboratories, internal report). Placebo capsules (P) contained starch.

Treatment

Twenty children between 1 and 16 yr of age (mean: 8.3 yr) and with a predisposition to upper respiratory tract infections were treated with either BV or P under double-blind conditions according to a randomization list. The capsules were of identical presentation. Each patient received one capsule daily in the morning before breakfast, the first ten days of each month, for three consecutive months.

The immunological and biochemical parameters were evaluated at presentation (day 0), during (day 30) and after (day 90) the treatment. Clinical examinations were performed on day 0, 30, 90 and 180.

Isolation of peripheral blood mononuclear cells (PBMC)

Blood samples were taken in the morning between 8 and 10 a.m. 20 – 30 ml of peripheral blood were diluted 1:4 with a 0.9% NaCl solution and layered on Ficoll Hypaque gradients (Boyum, 1968). After centrifugation (30 min, 800 × *g*, 20°C), the cells at the interface were harvested and washed three times with large volumes of ice-cold saline. Viability of the cells, determined by Trypan blue exclusion, exceeded 90%.

Cell characterization

The two main lymphocyte populations were identified by direct immunofluorescence. T-lymphocytes, T-suppressors and T-helpers were identified after staining for thymic antigens with FITC-labelled monoclonal antibodies (Leu 1, Leu 2a and Leu 3a from Becton-Dickinson, Sunnywale, Ca). B-cells were detected by direct immunofluorescence after staining of surface immunoglobulins (sIg) with TRITC-labelled F (ab')$_2$ fragments of polyvalent or monospecific anti-immunoglobulins (Winchester, 1976). Monocytes were identified by testing an aliquot of the isolated cells for non-specific α-naphtylacetate esterase. Briefly, 3 – 5 × 10^5 cells were incubated for 20 min at 37°C in a substrate solution of 0.1% pararosaniline, 4% sodium nitrite, and 1.2 mM α-naphtylacetate, buffered with phosphate to pH 5.9. Cells displaying a pronounced diffuse brownish reaction were counted as monocytes (Mueller, Brun del Re, Buerki, Keller, Hess & Cottier, 1975).

Mixed lymphocyte culture (MCL)

The method was that of O'Leary, Reinsmoen & Junis (1976). PBM cells were isolated by Ficoll centrifugation before being washed and suspended in RPMI-1640 culture medium supplemented with L-glutamine, penicillin-streptomycin and 20% pooled human AB serum from normal, untransfused male donors. The cultures were set up in sterile round-bottomed microplates (Nunc, Intermed, Denmark) and each stimulator-responder combination was performed in quintuplicate. 100 μl of the appropriate responder cells and 100 μl of the appropriate stimulator cells at the same concentration of 5 × 10^5 cells ml^{-1} were added to each well. The responder cells were from the patients, while the stimulator cells were derived from two normal donors and were irradiated with 1500 rad before culture. The cultures were incubated for 5 days at 37° in a 5% CO_2 atmosphere before addition to each well of 0.5 μCi methyl-^{3}H-thymidine (84.8 Ci $mmol^{-1}$, New England Nuclear, West Germany). Responder and stimulator cells were incubated separately as controls. 18 h after pulsing, the cells were harvested with a Titertek^R cell harvester (Flow Laboratories) and filtered on glass fiber filters. After washing, the filters were immersed in a scintillation fluid and the activity determined on a SL 4000 Intertechnique scintillation counter.

On day 0, 30 and 90, the patients' cells were stimulated with irradiated cells deriving from one of the two donors (always the same one). The patients' responses were then evaluated as percent of the mean response of the cells of four normal healthy donors to the same stimulator cells.

Enzyme assays

Enzymes were assayed on either intact or homogenized cells within 2 h after the gradient centrifugation. Cell homogenate was obtained from the cell suspension by freezing in liquid nitrogen and thawing three times. Assays were optimized for linearity of the reaction with respect to duration of incubation as well as to cells, protein content, and substrate concentration. *5'-nucleotidase* (EC.3.1.3.5.) was assayed on intact cells according to the method of Beaufay *et al.* (Beaufay, Amar-Costesec, Feytmans, Thinés-Sempoux, Wibo, Robbi & Bethet, 1974). Incubations were performed in duplicate at 37°C for 20 min in a total reaction volume of 4 ml. 0.2 ml of cell suspension were added to a mixture containing 50 mM Tris-HCl buffer at pH 7.5, 8 mM $MgCl_2$ and 2 mM 5'-AMP. The reaction was stopped with 30% ice-cold TCA. Inorganic phosphate was removed by centrifugation and then measured colorimetrically at

820 nm by the method of Chen, Toribara & Warner, (1956). *β-N-acetylglucosaminidase* (EC. 3.2.1.30.) was estimated on total homogenate by measuring at 405 nm the liberation of p-nitrophenol from the substrate *p*-nitrophenyl-*N*- acetyl-*β*- -glucosaminide. 0.2 ml of cell homogenate were incubated for 30 min at 37°C in a total volume of 0.7 ml containing 3.2 mM of substrate, 0.28 mM EDTA, 0.28 mM $NaHCO_3$, 0.003% Triton X, 0.03 M ethanol and 0.08 M Na-citrate, 0.08 M citric acid buffered at pH 4.5. The reaction was stopped by addition of 2.0 ml of an ice-cold solution containing 133 mM glycin, 83 mM Na_2CO_3, 67 mM NaCl. The specific activity was calculated by relating the optical density values to a standard curve of *p*-nitrophenol and expressed as *n*mole per hour per 10^6 cells or per mg of protein homogenate.

Protein determination

Proteins were determined according to the method of Lowry, Rosebrough, Farr & Randall (1951).

Statistics

Differences in enzymatic activities, surface markers, allogeneic responses and numbers of infections, were analyzed by the analysis of variance (one-way), the paired *t*-test and the rank-sum test. The correlation between MLC data and numbers of infections was tested by the rank correlation coefficient (Spearman).

RESULTS

The opening of the randomization code showed that eleven children were treated with BV and nine with P.

MLC

The MLC responses of BV- and P-treated patients are shown in Fig. 1. Apparently the patients' alloreactivity was depressed when compared to the response of normal healthy donors (controls) against the same stimulator cells. Before treatment the patients' response reached an average of 60% when the controls' response is equaled to 100%. It must however, be pointed out that the controls belonged to a different age group, being adult blood donors.

In Figs 2a and b, the specific immune reaction elicited by allogeneic cells in patients' PBMC at 30 and 90 days is confronted with the response at presentation. At day 30 (Fig. 2a) and 90 (Fig. 2b), the BV-treated patients displayed an improved responsiveness when challenged *in vitro* with the same alloantigens (same stimulator cells of day 0). In

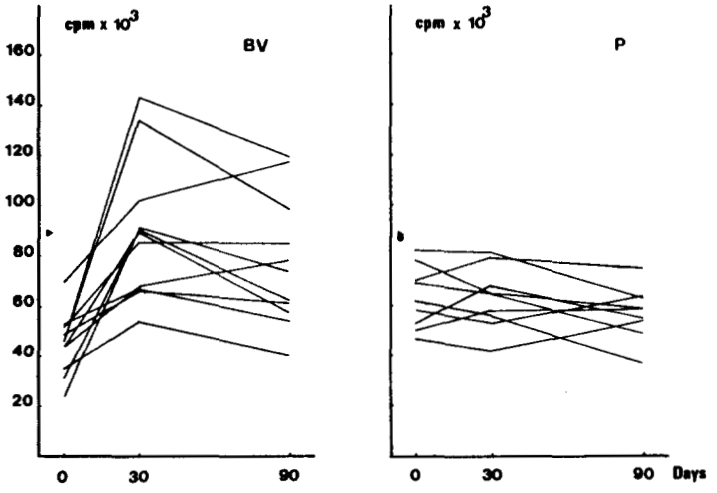

Fig. 1. One-way MLC response of BV- and P-treated patients. The incorporation of ^{3}H-thymidine by patients' PBMC after culture with irradiated allogeneic stimulator cells is expressed as counts per minute (cpm) for days 0, 30 and 90. Each patient was always confronted with mononuclear stimulator cells derived from the same donor. The level of the controls' response is indicated by the solid triangle (▲) and expresses the mean response of four healthy donors to the same donor's stimulator cells used to challenge the patients' PBMC.

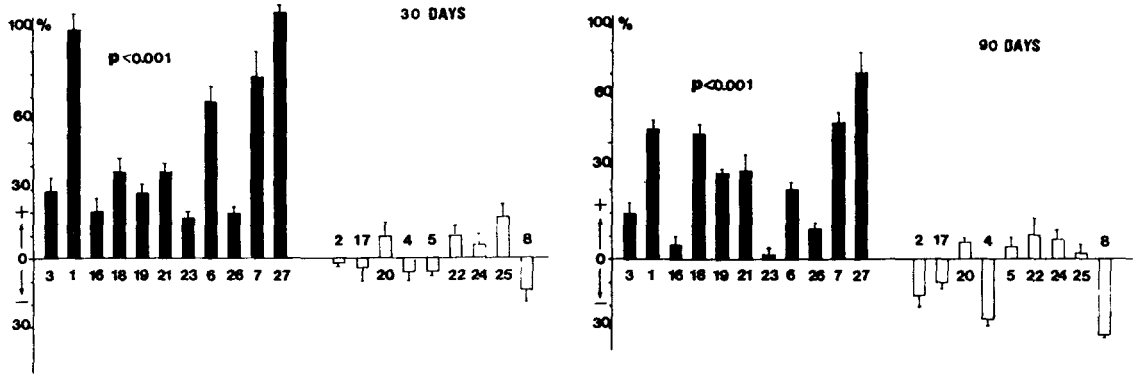

Fig. 2. Variation of MLC relative response at day 30 and 90 after BV or P administration. The patients' MLC relative response was calculated as percentage of the mean response given by healthy controls to the same stimulator cells. The variations of this response after 30 (a) and 90 (b) days of BV or P treatment are reported for each patient. Practically, the values of the response at day 0 (before treatment) were subtracted to the values recorded at day 30 and 90. The bars represent the registered differences and the underlying figures represent the number assigned to each patient by the randomized double-blind selection. ■ BV; □ P.

this group, the modification of the relative response was significant both at day 30 and at day 90 ($P < 0.001$). On the contrary, the P group did not show any significant modification at any time of the treatment.

Surface markers

Table 1 summarizes the relative distribution of mononuclear cells after staining for surface immunoglobulins, surface thymic antigens and esterase reaction in both groups of patients treated with either BV or P. The mononuclear cell fraction of both groups presents a similar cell composition at any time of treatment; no significant difference could be found.

Enzyme markers

The enzymatic characteristics of the mononuclear cell fraction isolated from both groups of patients are reported in Table 2. At any treatment time both groups revealed a quite similar activity level for the non-specific esterase as well as for the β-*N*-acetyl-glucosaminidase of monocytes. However, in both BV and P groups the 5'-nucleotidase, which is mainly associated with the plasmic membrane of lymphocytes, decreased during the treatment in a similar fashion (see Table 2).

Clinical observations

The clinical response of both BV and P groups is shown in Fig. 3 where the frequency of infectious episodes is reported for the same period of the year, during 6 months, before and after treatment. Before treatment the mean frequency of infectious episodes was 9.2 (1.53/month) and 9.7 (1.61/month) in BV and P group respectively. Furthermore, the two groups of patients did not differ significantly with regard to type and severity of infections. The mean frequency of infections decreased to 2 episodes (0.33/month) after BV administration (−78.3%) and 5.4 (0.9/month) after P treatment (−44.3%). The difference between the two groups is statistically significant at $P < 0.05$.

Table 1. Relative distribution of surface immunoglobulins and surface thymus antigens in PBMN.

Treatment	Days	Surface immunoglobulins (%)			Thymus antigens (%)		
		sIgtot	IgM	IgG	Leu 1	Leu 2	Leu 3
P	0	13.0 ± 10.7	3.1 ± 3.0	3.6 ± 3.9	67.0 ± 9.4	21.4 ± 8.3	41.0 ± 12.3
	30	12.7 ± 8.5	2.1 ± 1.9	4.0 ± 5.5	60.6 ± 10.5	22.9 ± 10.2	34.0 ± 14.8
	90	14.2 ± 7.8	1.9 ± 1.4	5.7 ± 5.8	62.4 ± 7.4	23.1 ± 7.9	37.7 ± 9.9
BV	0	11.8 ± 6.3	2.6 ± 2.0	3.2 ± 2.6	58.6 ± 8.9	21.5 ± 6.8	36.5 ± 6.9
	30	14.2 ± 7.3	3.4 ± 4.0	5.3 ± 3.9	60.3 ± 7.1	21.8 ± 7.2	35.6 ± 8.3
	90	11.2 ± 7.5	1.6 ± 2.4	5.0 ± 4.1	63.5 ± 7.9	21.1 ± 7.3	41.6 ± 9.0

Table 2. Activity variations of marker enzymes in peripheral blood mononuclear cells

Treatment	Days	α-naphtylacetate esterase (% positive cells)	5'-nucleotidase (nmole/h/10^6 cells)	β,N-acetyl-glucosaminidase (nmole/h/10^6 cells)
P	0	12.2 ± 7.6	26.3 ± 10.7	3224.8 ± 672.4
	30	15.7 ± 13.1	21.7 ± 8.7	3360.8 ± 642.5
	90	11.7 ± 6.1	17.1 ± 3.0	3093.1 ± 419.0
BV	0	11.5 ± 7.6	17.8 ± 6.8	3316.6 ± 747.3
	30	9.6 ± 4.1	14.9 ± 6.5	3055.0 ± 528.6
	90	11.4 ± 8.9	12.3 ± 5.4	2926.4 ± 786.7

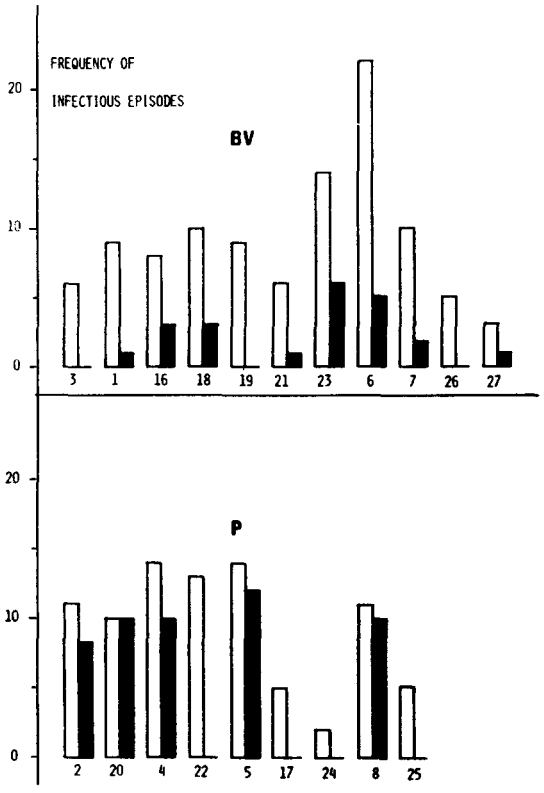

Fig. 3. Frequency of infectious episodes before and after BV or P treatment. The number of infectious episodes observed in the same period of the year during 6 months before and after BV or P therapy is reported. The infections considered were bronchitis, tonsillitis, pharyngitis, sinusitis, rhinitis and otitis. The number under the bars was assigned to each patient by the randomized double-blind selection. □: before treatment; ■ : after treatment.

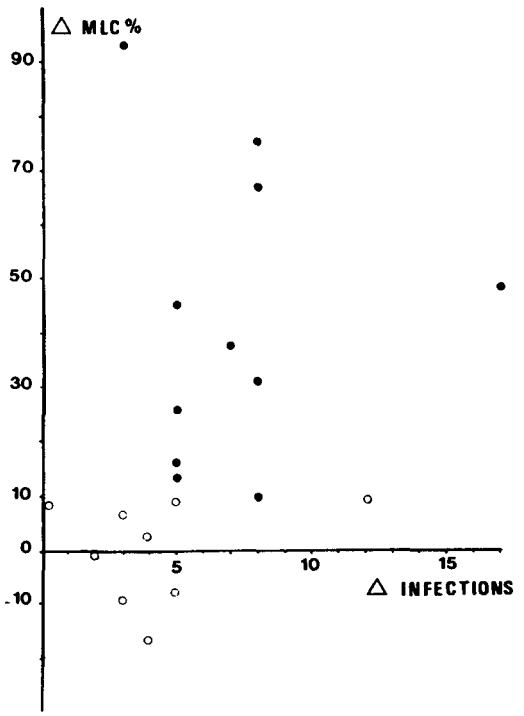

Fig. 4. Correlation between the mean variation of the MLC relative response and the decrease in infectious episodes after BV or P administration. For each patient the mean variations in MLC relative response (%△ MLC) is plotted against the difference in the number of infectious episodes (△ infections) before and after BV or P treatment. △ MLC was calculated as follows:

$$\%\Delta MLC = \frac{\text{MLC relative response day 30 + MLC relatives response day 90}}{2} - \text{MLC relative response day 0.}$$

Spearman rank correlation coefficient = 0.54, $P < 0.01$.
O : P; ● : BV.

Most interesting, the mean variation of the MLC response after BV or P administration correlated positively with the difference in the number of infectious episodes (Fig. 4). The correlation is statistically significant as calculated by the Spearman rank correlation coefficient ($r = 0.54$, $P < 0.01$).

DISCUSSION

Alloreactivity is the unique parameter significantly affected in PBMC of patients treated with BV. This speaks for a general stimulation of cell-mediated immunity and is in line with data reported by Clot & Andary (1980) and Girard & Fleury (1979), which showed an increased response to lectin stimulation of mononuclear cells from BV treated patients. In another investigation, Puigdollers *et al.* (1980) found an augmented antibody production in patients receiving BV. All the other immunological and biochemical parameters did not show any clear modification, but the enzyme 5′-nucleotidase deserves nevertheless a short notice. This enzyme, essentially located on the plasma membrane of B- and T-lymphocytes, is involved in the purine metabolism in relation to the immune response and appears to participate in lectin-mediated transformation and proliferation of lymphoid cells (Edwards, Gelfand, Burk, Dosh & Fox, 1979; Rowe, De Gast, Platts-Mills, Asherson, Webster & Johnson, 1980; Blatt, Reaman & Poplack, 1980). It has been shown that in murine PBMC stimulated by lectins as well as in chemically-induced tumor cells the level of 5′-nucleotidase decreases (Dornand, Rémissiac & Mani, 1977; Raz, Collard & Inbar, 1978). In addition, similar results were obtained with human PBMC stimulated by Con A (Losa, unpublished). Very low levels of 5′-nucleotidase were also found in certain types of leukemia and immunodeficiency diseases (Losa, 1982). Although we report here a decrease in 5′-nucleotidase activity in both BV- and P-treated groups (Table 2), these variations have probably no particular significance, being well contained within the normal range of activity (Losa, Morell & Barandun, 1982). This might further indicate that no patient suffered from a real immunodeficiency when this is defined by alterations of the following parameters, i.e. decrease of circulating B lymphocytes and impairment of antibody production (Losa *et al.*, 1982), alterations of T-cells populations (Cohen, Mansur, Dosch & Gelfand, 1980), disturbances of enzymes involved in the purine metabolism (Rowe *et al.*, 1980).

In our opinion, BV may exert its action by physiologic stimulation without a direct mitogenic or antigenic effect, as is also evident from the fact that the PBMC composition was unaffected (Table 1). However, one cannot exclude a direct antigenic effect of the bacterial extract (Clot & Andary, 1980) even if no convincing evidence is available that this type of immunostimulation is occuring *in vivo*. In short, the mechanism by which BV exerts its action is unknown. Still, we can reasonably exclude an effect on PBM phagocytes since the activity level of the lysosomal enzyme β-*N*-acetyl-glucosaminidase and the number of monocytes remained unchanged during the treatment.

Last but most important, BV proved to be clinically effective. In fact, the frequency of recurrences after BV treatment was significantly ($P < 0.05$) lower than that in the P group (Fig. 3). In addition, the clinical response correlated positively with the MLC data. As shown in Fig. 4, a small difference in the infectious episodes estimated before and after treatment corresponds to a small increase or, in some cases, even to a decrease in the MLC relative response of the P-treated patients. On the contrary, BV patients showed an increased MLC response corresponding to a higher difference in the number of infectious episodes. In fact, the Spearman rank correlation coefficient $r = 0.54$ is significant at the level $P < 0.01$. Taken together, these results suggest that BV has the capacity of restoring functional properties of normally distributed but underfunctioning components of the immune system and that its pharmacologic action is exerted through a functional and harmonious modulation of the immune system as a whole, without specifically affecting a particular cell component. This remarkable effect which reaches its maximum at day 30 might depend on the route of administration. Thus, it is conceivable that the orally administered BV first exerts its stimulation through the gut-associated lymphoid tissue (Owen, 1977) and that the activated cells spread afterwards in the organism by the lymphatic circulation (McDermott & Bienenstock, 1979). The proved effectiveness together with the absence of any toxic or collateral effect point out BV as a drug of choice for correcting functional disorders of the immune system.

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