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COMPENSATION OF CYCLOPHOSPHAMIDE IMMUNOSUPPRESSION BY A BACTERIAL IMMUNOSTIMULANT (BRONCHO-VAXOM) IN MICE

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ABSTRACT

The compensatory effect of a bacterial lysate, Broncho-Vaxom (BV) on the immunosuppressive action of cyclophosphamide (CY) was investigated. In CY immunosuppressed mice, BV treated animals recovered to normal levels of IgM and IgG in serum as well of IgA and IgG in gut secretions significantly earlier than controls. Furthermore, normal cell proliferation in thymus, as estimated by measuring the relative size of this organ was achieved earlier in BV treated mice than in control mice. Oral treatment with BV restores the number of IgM anti SRBC producing cells in spleen, in CY immunosuppressed mice. Since immunosuppression induced by CY increases the susceptibility to various infections, we tested in immunosuppressed animals the protective effect of BV towards IP challenge infections with *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Klebsiella pneumoniae* var *ozaenae*, *Pseudomonas aeruginosa* and *Candida albicans*. BV led to an enhanced resistance towards both pneumococci and staphylococci challenge infections but not to the other challenge microorganisms.

INTRODUCTION

Broncho-Vaxom (BV), a bacterial lysate consisting of twentyone bacterial strains corresponding to eight bacterial species frequently associated with respiratory tract infec-

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tions, has been proved to restore several immunological parameters in children with IgA deficiency (1). Moreover, it has been found to stimulate in healthy volunteers the production of secretory IgA in saliva, and of IgG and IgM in serum (2). Also, oral administration of BV to mice led to an increase in the IgA levels in both intestinal and pulmonary secretions (3).

In order to investigate the effect of BV on the recovery from immunodeficiency, we planed a model for the study of immunosuppression in mice considering the similarity with the pattern occurring in humans. Cyclophosphamide (CY) was chosen among other immunosuppressive drugs because of its prime effect on B-lymphocytes and consequently on Igs levels (4).

MATERIALS AND METHODS

Animals

Female Swiss mice weighing 20-22 g were obtained from the "Instituto Central de Animales de Laboratorio". Daganzo, Spain.

Broncho-Vaxom

Broncho-Vaxom (BV) is an immunobiotherapeutic preparation (OM Laboratoires, Geneva, Switzerland). It is a lyophilized bacterial lysate of *Haemophilus influenzae, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus viridans,*

Klebsiella pneumoniae, Klebsiella ozaenae, Staphylococcus aureus and *Neisseria catarrhalis (Branhamella catarrhalis)*.

Broncho-Vaxom treatments

In studies of the evolution of immunological parameters after immunosuppression, mice received daily for 5 days an oral dose of 4.66 mg of BV diluted in 0.2 ml of saline. The same number of immunosuppressed control mice received the same treatment with saline. Schedule of treatments is indicated in each table of results.

In the protection towards experimental infection studies, mice received a daily oral dose of 2.33 mg of lyophilized BV diluted in 0.2 ml of saline for 10 days prior to CY intraperitoneal inoculation, that was administered 10 days after the end of BV treatment and 5 days before the challenge infection. The same number of control mice received saline instead of BV. The number of mice used is indicated in each table of results.

Immunosuppression

Mice received each a single intraperitoneal dose of 200 mg/kg of cyclophosphamide (CY) (Genoxal, Funk Laboratories, Barcelona, Spain) diluted in 0.2 ml of saline.

Determination of Igs levels

Groups of 10 mice were scheduled and processed as a single unit. Once the treatment ended, at intervals of time indi-

cated in results, mice were sacrificed by cervical dislocation and their organs and blood were immediately removed and placed at 4°C. Igs determinations were performed as previously described (3). Briefly, guts were thoroughly rinsed with saline containing sodium azide at a final concentration of 1 g/l. Their total content and rinsing solution were centrifuged twice at 8000 x G for 15 minutes at 4°C in order to remove particulate material. Supernatants were 100 fold concentrated by negative pressure ultrafiltration using 8/32 visking tubing membranes (Scientific Instruments Center Ltd., London, England) and then kept frozen until analyzed. Blood samples were collected by cardiac puncture and the sera kept frozen until the immunoglobulin assay. IgA, IgG1, IgG2a, IgG2b and IgM determinations were performed by quantitative radial immunodiffusion (5) using commercial plates (Meloy Lab. Inc. Springfield, Va. USA). Ig concentrations are given with respect to mg of protein in the concentrated samples. The protein content was determined by the Lowry's method (6).

Determination of splenic and thymic indexes

Splenic and thymic indexes were calculated dividing the organ weight in milligrams by the body weight in grams. Increase in the size of spleen and thymus may be considered as an indication of lymphoid cell proliferation (7).

Determination of direct PFC

At the time intervals indicated in results, mice were killed and their spleen removed in order to count the

number of anti SRBC direct (IgM) PFC according to Cunningham's method (8). Briefly, spleens were dissected to prepare a single cell suspension in PBS. Then, a mixture of 20 µl of an appropiately diluted cell suspension, 20 µl of normal guinea pig serum (Institut Pasteur, París), 20 µl of a SRBC suspension in PBS ($\simeq 3.5 \times 10^8$ cel/ml) and 20 µl of PBS were incubated at $37^{o}C$ for 1.5 hours in a Cunningham's chamber for the development of PFC.

Challenge infections

Mice treated as indicated above were challenged each with an intraperitoneal inoculation of either *Staphylococcus aureus* strain 547, *Streptococcus pneumoniae* strain CFLN, *Klebsiella pneumoniae* var *ozaenae* strain 5050, *Pseudomonas aeruginosa* strain CBC110 and *Candida albicans* strain E1, *S. aureus* 547 and *K. pneumoniae* 5050 are included in the lysate. Infective doses were around the LD50.

RESULTS

Effects of Broncho-Vaxom on cell proliferation in spleen and thymus in immunosuppressed mice.

Immunosuppression with CY resulted in a significant reduction of the relative size of both spleen and thymus 5 days after the IP administration of the drug (Table 1). The size of these organs recovered in both BV treated and non treated CY immunosuppressed mice. Such recovery may be considered as an indication of the restoration of cell

TABLE 1

Effects of the oral administration of Broncho-Vaxom (BV) on the size of spleen and thymus in cyclophosphamide immunosuppressed (IS) mice.

Day [a]	Ratio $\frac{IS + BV}{IS}$ [b]		Ratio $\frac{IS}{Normal}$ [c]	
	Splenic index	Thymic index	Splenic index	Thymic index
5	-	-	0.29	0.44
15	0.89	1.27	1.4	1.09
20	1.2	1.42	0.9	0.86
25	0.99	1.19	1.12	0.86
30	1.3	1.56	1.07	0.68
40	1.7	1.55	0.83	0.68

a) At day 0 animals were treated with CY. From days 5 to day 10 they received BV oral treatment.

b) Ratio $\frac{IS + BV}{IS}$ means the quotient between the mean value of ten immunosuppressed mice treated with BV and the mean value of ten immunosuppressed mice.

c) Ratio $\frac{IS}{Normal}$ means the quotient between the mean value of ten immunosuppressed mice and the mean value of ten normal mice.

proliferation in spleen and thymus, which are definitely implicated in the immune response. No significant differences were observed in the recovery of spleen size in BV treated and in non treated mice, but BV-treated mice showed a significantly ($p < 0.05$, Student's t test) faster recovery of thymus size than in BV non-treated controls (Table 1).

Effects of Broncho-Vaxom on the Igs levels in immunosuppressed mice.

Treatment with CY caused a very important reduction in the concentration of the different classes of Igs, both

TABLE 2

Effects of the oral administration of Broncho-Vaxom (BV) on Igs levels in serum in immunosuppressed (IS) mice.

Day [a]	Ratio IS + BV / IS [b]		Ratio IS / Normal [c]	
	IgM	IgG	IgM	IgG
5	-	-	0 [d]	0.06
15	8.00	3.00	0.10	0.15
20	1.37	1.50	0.80	0.38
25	0.91	0.97	1.20	0.50
30	1.10	1.50	1.00	0.68
40	0.91	1.40	1.10	0.60

a), b) and c) as in table 1.

d) No IgM was detected by our methods 5 days after CY treatment.

TABLE 3

Effects of the oral administration of Broncho-Vaxom (BV) on Igs levels in gut secretions in immunosuppressed mice.

Day [a]	Ratio $\frac{IS + BV}{IS}$ [b]		Ratio $\frac{IS}{Normal}$ [c]	
	IgA	IgG	IgA	IgG
5	-	-	0.050	0.057
15	0.80	1.30	0.50	0.17
20	1.20	0.65	0.78	0.71
25	1.50	1.56	0.37	0.45
30	1.70	1.80	1.20	0.77
40	2.40	1.50	0.40	0.54

a), b), c) as in table 1.

in serum and gut secretions, 5 days after CY intraperitoneal administration (Tables 3 & 4). Recovery of Ig levels was studied in BV-treated immunosuppressed mice and compared to those of immunosuppressed controls (Tables 2 and 3). A clear effect of the orally administered bacterial lysate on the recovery of IgM levels was observed in serum. Early after BV treatment, the differences between treated and non treated animals were highly significant ($p < 0.01$, Student's t test) (Table 2). Moreover, we were able to observe that the recovery from the depression of serum IgG

TABLE 4

Effects of the oral administration of Broncho-Vaxom (BV) on PFC anti SRBC in spleens in immunosuppressed mice.

Day [a]	Ratio $\frac{IS + BV}{IS}$ [b]		Ratio $\frac{IS}{Normal}$ [c]	
	PFC/spleen	PFC/10^8 /lymphocytes	PFC/spleen	PFC/10^8 /lymphocytes
7	-	-	0.16	0:25
11	4.4	2.4	0.30	0.38
13	4.4	5.0	0.22	0.30
15	2.5	2.3	0.66	0.60
20	0.99	0.86	0.84	1.5

a) At day 0 mice were treated with CY. From day 2 to day 7 they received BV oral treatment.

b) Ratio $\frac{IS + BV}{IS}$ means the quotient between the mean value of six immunosuppressed mice treated with BV and the mean value of ten immunosuppressed mice.

c) Ratio $\frac{IS}{Normal}$ means the quotient between the mean value of six immunosuppressed mice and the mean value of ten normal mice.

(IgG1, IgG2a, IgG2b) was significantly ($p < 0.05$ Student's t test) better in BV treated animals than in control mice. BV treated immunosuppressed mice recovered IgA and IgG levels in gut secretions, particularly IgA ($p < 0.05$, Student's t test), better than controls that only received CY (Table 3). The method in these studies did not allow IgM detection in gut, consequently the ratio between IgM levels of Broncho Vaxom treated and BV non-treated mice could not be established.

Effects of Broncho-Vaxom on the number of direct PFC counts in spleens of immunosuppressed mice.

Intraperitoneal administration of CY caused 7 days later a considerable reduction in the concentration of direct PFC against SRBC (Table 4). Recovery of direct PFC counts was studied in BV treated immunosuppressed mice and compared to that of immunosuppressed controls (Table 4). Recovery of normal PFC counts, expressed either as PFC per spleen or PFC per 10^8 lymphocytes, was achieved significantly ($p < 0.10$, Student's t test) earlier in BV treated animals than in those that only received saline (Table 4). BV treated mice reached normal values eleven days after CY treatment whereas non BV treated animals did not reach normal values until day twenty after they received CY (Table 4).

Effect of Broncho-Vaxom on the resistance to challenge infections in immunosuppressed mice.

In IP challenge infections in CY immunosuppressed mice, animals previously treated with BV were significantly more resistant towards challenge infections with both *Staphylococcus aureus* and *Streptococcus pneumoniae* specially towards the former (Table 5). No protection was observed when animals were challenged with either *Klebsiella pneumoniae*, or *Pseudomonas aeruginosa* or *Candida albicans*.

TABLE 5

Protective activity expressed as cumulative survival induced by BV in immunosuppressed mice infected IP with different microorganisms. Challenge doses corresponding to about LD_{50}.

Microorganism	Number of mice inoculated	%Survival		Protection on survival
		Treated	Controls	
Staphylococcus aureus 547	25	80	10	70%
Streptococcus pneumoniae CFLN	50	66	48	18%
Klebsiella pneumoniae 550	40	48	48	-2%
Pseudomonas aeruginosa	50	13	12	1%
Candida albicans	50	82	84	-2%

DISCUSSION

CY is an extensively used immunosuppressive drug that has been proved to affect mainly B-lymphocytes and consequently the levels of Igs (10). In our experimental model, Igs levels decreased very significantly in both sera and secretions 5 days after CY treatment, as it has been described for other immunological parameters and other immunosuppressive agents (11). Therefore, amid other side effects, CY treatment might increase the risk of infection through mucosal tissues. Our results show that BV administration after CY immunosuppression helps to restore the levels of Igs, both in serum (IgM and IgG) and gut secretions (IgA). The modulation by Broncho Vaxom of immunoglobulin levels both in serum and secretions in immunosuppressed animals and in the levels of IgA in secretions in normal animals (3) is of sufficient magnitude to suggest polyclonal activation.

Oral administration of BV causes an increase in the number of direct PFC anti SRBC as single doses of well established polyclonal activators do (11, 12, 12), thus confirming that at least part of the response induced by BV is non specific.

The effect of BV on the Igs levels and on direct PFC suggests that Broncho-Vaxom acts "in vivo" as a B-lymphocyte polyclonal activator. In addition, the

recovery of normal thymus size, such as observed after BV treatment in these studies, suggests that this bacterial immunopotentiator may also stimulate T-lymphocyte proliferation. Whether B-lymphocytes activation is mediated by T-lymphocytes can not be infered by the experiments herein described.

Unlike the immunological parameters above described, the induction of enhanced resistance towards intraperitoneal challenge infections, appears to be specific, since a significant increase in resistance can be observed against *Staphylococcus aureus* 547 and *Streptococcus pneumoniae* CFLN, the first being used in Broncho-Vaxom and the second corresponding to a species included in it, whereas no enhanced resistance was observed against non related microorganisms. We can not explain why there was no enhanced non especific resistance to infection when immunological parameters suggest non specific stimulation. However, different results have been reported for other non specific immunostimulants depending on route and timing of challenge infections (14, 15, 16). There is no enhanced resistance either towards challenge infection with strain 5050 of *Klebsiella pneumoniae* which is included in the lysate. Therefore we can not conclude either that Broncho-Vaxom does not enhances non specific resistance to challenge infections or that the enhanced resistance against both staphylococcus and pneumococcus is due to specific stimulation.

Although the results corresponding to immunological parameters and the ones corresponding to enhanced resistance look contradictory, it has to be considered that Broncho-Vaxom contains mainly immunomodulators but also antigens of bacterial origin, so both specific and non s pecific responses can be expected to be induced.

Since BV restores immunoglobulin levels when administered after cyclophosphamide immunosuppression and confers protection when administered before some challenge infection, it can be concluded that BV prevents some of the effects of immunosuppression. A great deal of importance has been given to IgA as one of the main barriers against infections through mucosal surfaces (17, 18, 19). Since BV has been described to increse the levels of IgA in secretions in normal man as well as in normal and immunosuppressed mice, and at the same time, to decrease recurrence of respiratory diseases in IgA deficient children (1), it can be assumed that Broncho Vaxom has a therapeutic value in the prevention of infections through respiratory mucosal both in normal and immunosuppressed men.

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