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Suppression of Local and Systemic Responses in Streptococcal Cell Wall-Induced Acute Inflammation of the Air Pouch by Cyclosporine A

Comparison with the Effects of Two Anti-Inflammatory Bis-Benzimidazoles

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Injection of streptococcus group A cell wall-derived peptidoglycan polysaccharide into a subcutaneous air pouch causes local outpouring of neutrophils and macrophages and distant hemopoietic proliferation in spleen and bone marrow. Cyclosporine A (CyA) suppressed neutrophil accumulation and all cell lines of hemopoiesis. trans-1,2-Bis(5-amidino-2-benzimidazolyl)ethene (BBE) also interfered with neutrophil exudation, yet reduced only the erythroid component of the hemopoietic process. The ethane analogue of BBE, on the other hand, did not prevent neutrophil emigration, but held down splenic erythropoiesis and myelopoiesis. All three compounds stimulated streptococcus group A cell wall-derived peptidoglycan polysaccharide uptake by pouch macrophages, CyA being the least active. BBE and its ethane analogue also produced a shift of wear-and-tear pigment from large numbers of small splenic macro-phages into small numbers of large macrophages. The pouch model is very useful in the study of anti-inflammatory compounds and has furnished the first evidence of CyA interference with massive neutrophilic infiltration and with hemopoietic signals. (Am J Pathol 1993, 142:1227–1237)

In recent years novel bis-benzimidazoles have been recognized as effective anti-inflammatory agents.[1–3] They were tested in the Lewis rat, which responds to the systemic application of streptococcus group-A cell wall-derived peptidoglycan polysaccharide (PG-APS) with arthritis and with characteristic neutrophil-assisted osteoclastic bone destruction and extensive pyogranulomatous lesions in spleen and liver. Both of the latter reactions were found to be uniquely susceptible to inhibition by *trans*-1,2-bis(5-amidino-2-benzimidazolyl)ethene (BBE), one of many compounds investigated.[3] The drug appears to block selectively the accumulation and activities of macrophages, be it the modified macrophages of the osteoclast type or the cellular components of parenchymal granulomata. In addition, it prevents the association of neutrophils with the osteoclasts and the influx of neutrophils into the granulomata. A further distinguishing property of BBE is its ability to stimulate splenic and hepatic macrophages to take up excessive amounts of PG-APS and thus to become greatly bloated.

To gain more information on its mode of action, BBE has now been studied for its influence on the events following the injection of PG-APS into a subcutaneous air pouch in the Lewis rat. In these experiments the streptococcal cell wall material is not disseminated throughout the body as it is in the arthritis model. Thus, any lesions encountered in sites other than the pouch must be interpreted as secondary responses to signals emanating from the pouch inflammatory cells.

Reaching beyond BBE, two additional compounds were included in the investigation. One was 1,2-bis-(5-amidino-2-benzimidazolyl)ethane (BBE-d). It was

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added to evaluate the importance of the central double bond for BBE activity. The other compound was cyclosporine A (CyA), which is similar to BBE in its antigranulomatous activity in the arthritis model[4] and for which interesting new properties were discovered.

Materials and Methods

Drug Sources

BBE and its single-bond derivative BBE-d were synthesized according to previously established procedures.[5] Purity was assessed by nuclear magnetic resonance spectroscopy, high-pressure liquid chromatography and elemental analysis (CHN). By those criteria BBE was 100% pure and BBE-d was 95% pure. CyA was a product of Sandoz Pharmaceuticals Corporation (East Hanover, NJ). Cremophor EL, the vehicle for CyA, was a gift from the Sandoz Research Institute (East Hanover, NJ).

Streptococcal Cell Wall Fragments

PG-APS was used to stimulate inflammation in the air pouches. The material was prepared from group A, type 3, strain 58 streptococci by a previously described method.[6] The bacteria were broken up in a Dyno Mill (Glenn Mills, Inc., Maywood, NY) at 4°C. Subsequently, glass beads and any remaining intact cocci were removed by filtration and centrifugation. The cell fragments were collected by centrifugation for 30 minutes at 30,000 × *g*. The material was washed and extracted three times at 56°C with 2% sodium dodecyl sulfate in phosphate-buffered saline (pH 7.0). Following further washes, initially with phosphate-buffered saline and then with distilled water, the cell wall fragments were lyophilized and kept at 4°C until use.

For application in the pouch experiments the cell walls were suspended in saline and sonicated for 35 minutes at 4°C. This was followed by 30 minutes of centrifugation at 10,000 × *g* and passage of the supernatant through a Millipore filter (0.45 μm) to eliminate any clumped cell wall fragments. The preparation was kept at 4°C until injection into the animals. Aseptic precautions were taken at all steps in the procedure.

Animals

Pathogen-free female Lewis rats of an average body weight of 200 g were obtained from Charles River, Raleigh, NC. They were kept on an ad libitum diet of Purina rat chow and water. An air pouch was induced under light ether anesthesia by the injection of 10 ml of bacterium-free air under the subcutaneous striated muscle of the back. During the course of a week the pouches were replenished twice with a few milliliters of additional air. At the end of the preparatory period the animals received an injection into the pouch of either 4 ml saline or saline containing 4.5 mg rhamnose equivalents of PG-APS. Treatment with BBE, BBE-d, or CyA was begun immediately afterward. BBE (10 mg/kg body weight) and BBE-d (5 mg/kg body weight) were given as single daily injections into the tail vein. The compounds were dissolved in 0.4 ml of pyrogen-free saline. Controls received saline only. CyA was given into the muscle of the hind legs as a single daily dose of 25 mg/kg body weight dissolved in 0.1 ml of Cremophor EL solution. The respective controls received vehicle only. All injections were carried out under light ether anesthesia, the last injections being given on the day before sacrifice. At specified times the rats were killed by an overdose of carbon dioxide. For a comparison of the effectiveness of the three compounds one might note that the daily doses of BBE, BBE-d, and CyA on a molar basis had a ratio of 1:0.5:1.

Histological and Hematological Studies

At autopsy of the animals blood was withdrawn from the heart, anticoagulated with potassium EDTA, and then used in a model 150 Baker cell counter to determine the white blood cell count, the red blood cell count, and the hematocrit and hemoglobin values. Blood smears were prepared for differential white blood cell counts.

The fluid contents of the air pouches were aspirated. The volume was measured, and total and differential white blood cell counts were performed. Histologic examination was carried out on the air pouches, spleens, livers, and knee joints, the latter including part of the distal shaft of the femur and the proximal shaft of the tibia. All specimens were fixed in neutral formalin, and the bones were decalcified before embedding. The tissues were stained routinely with hematoxylin and eosin and with the periodic acid Schiff method (PAS) after diastase digestion. Selected sections were also stained for iron with Turnbull's Blue and for lipofuscin with a prolonged Ziehl-Neelsen procedure.

To determine the area of macrophages in pouch tissue sections, the PAS-stained slides were placed under the 40× objective on a Nikon Microphot-FXA

microscope, and the resulting image was captured by using an FG-100-AT image processor (Image Technologies, Inc., Woburn, MA). The picture was displayed on a Sony Trinitron Super Fine Pitch color television monitor. By employing the Image Measure/IP IM 2500 Morphometry System (Phoenix Technology, Festival Way, WA) running under Image-Pro II software (Media Cybernetics, Silver Springs, MD) and using a mouse-type device controlling a video cursor, morphometric measurements of the cell areas were taken. The entire system was run on an IBM AT computer.

Statistical Analyses

Student's *t*-test was used to determine the significance of the difference between two means.

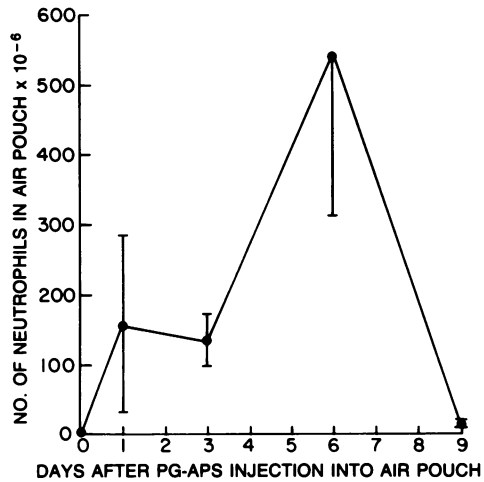

Figure 1. *Total number of neutrophils removed from the pouch lumina at various times after PG-APS injection. There was no drug treatment. Each point represents mean ± SEM of 4 animals.*

Results

The Pouch Reaction: Primary and Secondary Events

The repeated injections of air into the dorsal subcutaneum of the Lewis rat led to the stereotyped production of a pouch in the deep fascia. By the end of 1 week its wall consisted of 15 to 20 layers of fibroblasts peripheral to which there was a zone of vascularization including capillaries, venules, and arterioles. A few lymphocytes were scattered singly throughout the tissue or grouped around small vessels. Within a day after the introduction of PG-APS into the pouch there was a significant acute inflammatory reaction characterized by an infiltration of neutrophils and macrophages in the innermost layers of the wall. The majority of those neutrophils were in transit and moved on into the pouch lumen. The volume and white blood cell count of the purulent exudate peaked by day 6 after PG-APS injection and then decreased rapidly over the following three days (Figure 1). In contrast to the neutrophils, the macrophages remained in the pouch wall in much greater numbers. They acquired increasing amounts of PG-APS and could be easily identified histologically because of the PAS staining of the ingested material. The percentage of macrophages in the pouch exudate did not exceed 10% of the total white cell count. It should be noted that the neutrophils also took up PG-APS, albeit in small amounts. The inflammatory process led to further thickening of the pouch wall by stimulating capillary and fibroblastic proliferation, which reached its height on day 3 after PG-APS injection. Throughout the course of the experiments lymphocytes remained scarce in the pouch tissue. A few small groups of perivascular plasma cells were observed 9 days into the pouch inflammation.

At the time that the inflammation ran its course in the air pouch, striking changes also took place in the spleen. In the red pulp there developed extensive hemopoiesis, including proliferation of myeloid, megakaryocytic, and erythroid elements. From the graphs in Figure 2 it can be seen that the megakaryocyte counts on transverse sections of the spleens closely paralleled the degree of involvement of the red pulp, and the counts can therefore serve as general measure of the hemopoietic activity. Stimulation was noticeable as early as 1 day after PG-APS injection into the air pouch. The most

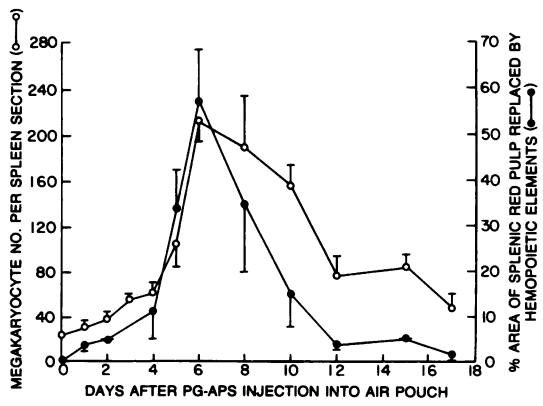

Figure 2. *Splenic megakaryocyte counts and extent of hemopoietic replacement of the red pulp at various times after PG-APS injection into the pouch. There was no drug treatment. The groups were made up of 4 animals. Megakaryocytes were counted on two sections of each spleen. Values are means ± SEM.*

rapid increase occurred on days 4 to 6, while beyond day 6 the values began to decline and had returned to the baseline by day 17. As could be expected, hemopoiesis was temporarily enhanced also in the bone marrow, causing a complementary reduction in adipose tissue. It should be noted that in rats with PG-APS-injected pouches none of the material was carried away beyond the confines of the pouch wall. This differed from the experience with the intraperitoneal route of administration, in which case PG-APS was removed by macrophages from the abdominal cavity and transported to liver as well as to spleen.

Effect of BBE, BBE-d, and CyA on the Pouch Lesions

As detailed in Materials and Methods, drug treatment began immediately after the application of PG-APS into the air pouch and consisted of single daily doses of the compounds, the last one being given on the day before sacrifice. Testing of each drug involved four groups of animals. Two groups received PG-APS and either drug or vehicle. Two more groups received saline and either drug or vehicle. In the following, use of the unspecified term "controls" refers to animals that had received PG-APS but no drug. Already early in the experiments it was obvious that CyA and BBE had a beneficial effect on the pouch reaction, and this became more pronounced as the disease advanced. Under the influence of those two drugs the pouches remained flaccid and collapsed, while in the controls they progressively enlarged and felt very tense. At the height of the pouch response, 6 days after PG-APS injection, about 2 to 4 ml of pus could be aspirated in the controls. No fluid could be removed from the CyA animals, and only a trace could be obtained from the BBE group. With BBE-d, on the other hand, the amount of exudate virtually matched the controls. Table 1 allows a comparison of the results in terms of numbers of white blood cells present in the pouches. For all controls and for the BBE-d group the percentage of neutrophils in the exudate was about 90%, macrophages accounting for the rest. In the BBE group macrophages made up about 50% of the very low total white blood cell count. Histological examination of the pouches in the CyA and BBE animals showed only rare small collections of neutrophils in the inner pouch wall and in the pouch lumen, markedly less than were seen in the controls and in the BBE-d group (Figure 3).

Table 1. *Number of Neutrophils in Air Pouch Lumina of Drug-Treated Rats and of Their Respective Vehicle-Treated Controls*

Treatment*	No. of neutrophils per pouch ($\times 10^{-6}$)†	
	Controls	Drug-treated
BBE	541 ± 508	1.3 ± 3.3‡
BBE-d	361 ± 232	356 ± 121
CyA	369 ± 282	0

* The rats were killed 6 days after PG-APS injection into the pouch. n = 5 or 6.
† Values are means ± SD.
‡ $P < 0.05$.

While with the neutrophils their total number in the pouch was of chief interest, with the abundantly present macrophages in the wall it was their variation in size that drew the most attention. As early as 3 days after PG-APS injection it was histologically evident that the cut surface areas of the macrophages were larger in the BBE and BBE-d groups than in their respective controls or in the CyA group. By day 9 the values had become stabilized, and the difference had become easily discernible on microscopic examination (Figure 4). Morphometric measurements at that time revealed that in the BBE and BBE-d groups the macrophages had about twice the area size as in the controls (Figure 5). Taking into consideration the constant size of the macrophage nuclei, one could calculate that the cytoplasmic volumes of the cells had about tripled. The increase in volume was due to a marked increase in ingested PG-APS. Analysis of the morphometric data of the CyA-treated animals also revealed a statistically significant increase in cell size. However, the compound was distinctly less active than either BBE or BBE-d, whether doses were compared on a weight or molar basis.

Effect of Drug Treatment on Splenic Hemopoietic Hyperplasia

The influence of the drugs on PG-APS-induced, pouch-dependent hemopoietic hyperplasia in the spleen was judged by the megakaryocyte count, the overall degree of occupancy of the red pulp by the proliferating cells and by evaluation of the prevalence of the three cell lines. The megakaryocyte counts were obtained generally on day 6 and day 9 after PG-APS injection where high values could be expected. For BBE the two groups of PG-APS-free controls for the 6-day time point were not available and were replaced by the 7-day values. This did not interfere with the interpretation of the data. As can

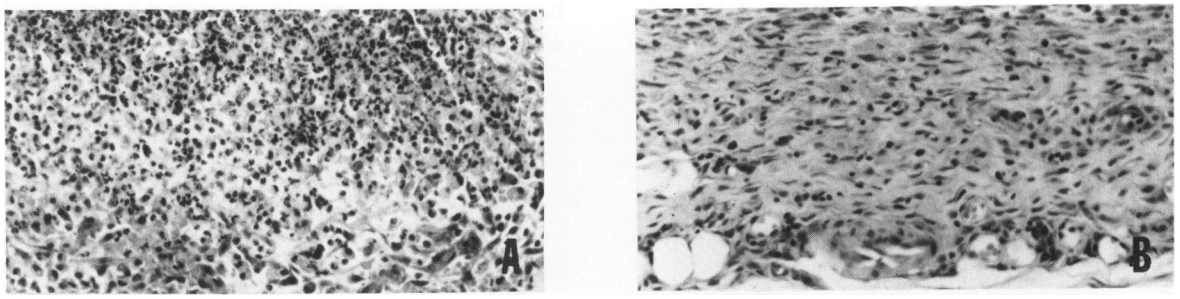

Figure 3. *Wall of air pouch 6 days after injection of PG-APS.* **A,** *heavy neutrophilic infiltrate in saline-treated control.* **B,** *no inflammation in animal treated with daily injections of BBE. H&E, ×162.*

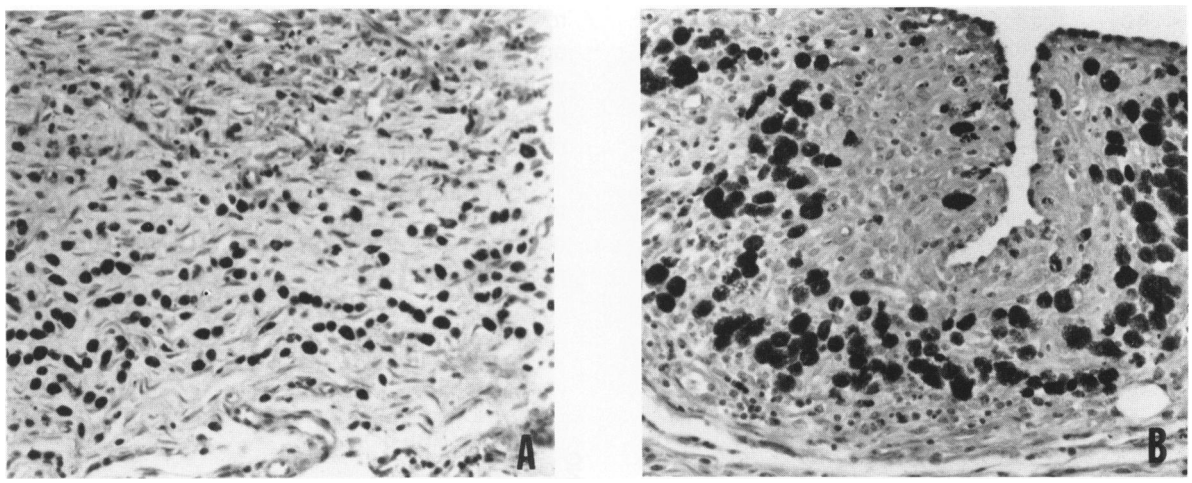

Figure 4. *PG-APS-laden pouch macrophages 9 days after PG-APS injection.* **A,** *small macrophages in saline control rat.* **B,** *enlarged macrophages in BBE-treated animal. PAS digest, ×162.*

be seen in Figures 6A and 6B, neither BBE nor BBE-d had any significant effect on the megakaryocyte count. There was no reduction in PG-APS-elicited megakaryocytic hyperplasia, and there was also no decrease in the baseline count in animals that had not received PG-APS. For CyA there was quite a different response. The megakaryocytic hyperplasia was completely eliminated, and even the baseline values fell significantly further (Figure 6C).

In agreement with the megakaryocyte data there was complete absence in the spleen of myeloid and erythroid precursors in the CyA-treated animals (Figure 7). In the BBE-d series there was suppression of the two cell lines on day 6, the red pulp occupancy rate being down to 5% from the controls' 60%. However, on day 9 there was no longer any discernible difference between the BBE-d-treated group and the control group. For the BBE group it was of interest that myeloid precursors were plentiful in the red pulp, while no erythroid elements were encountered (Figure 8).

Drug-Induced Stimulation of Splenic Macrophages

The spleens of all rats used in these experiments had easily identifiable yellow pigment deposits within groups of macrophages in the red pulp. The pigment was in the shape of granules or small blocks which assumed an orange color with the PAS digest procedure. It also was acid-fast and stained heavily with the iron stain. This combination of reactions sets it apart from PG-APS and identifies it as wear-and-tear pigment. The material became of interest when it was noted to be redistributed in animals that had received BBE or BBE-d, but not in those that had received CyA. Instead of being present as small collections in the cytoplasm of many macrophages, it was now concentrated as large deposits in only a few macrophages (Figure 9). It should be noted that the reaction occurred whether the animals had also been given PG-APS or not. No changes took place in the Kupffer cells

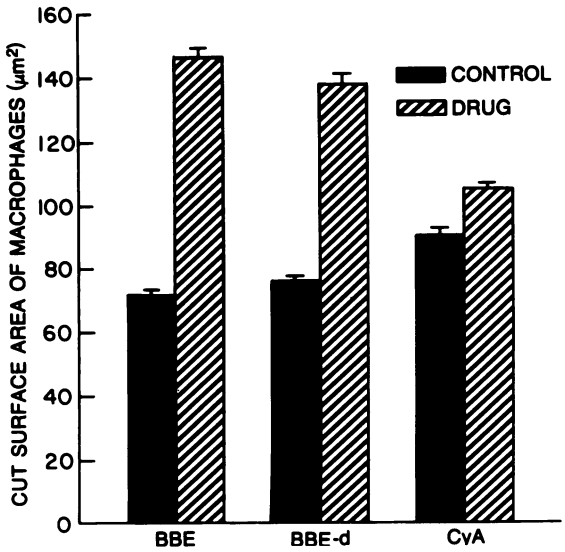

Figure 5. *Increased cut surface area of pouch macrophages in rats treated with BBE, BBE-d, or CyA. The experimental groups comprised 5 or 6 animals which were killed 9 days after injection of PG-APS into the pouch. In each animal 100 or 200 cells were measured. Values are means ± SEM. $P < 0.001$ for each of the three sets.*

because they were free of any stored material that could have been rearranged.

Effect of Drug Treatment on Hemopoiesis in the Bone Marrow and on Circulating White Blood Cell and Hemoglobin Values

The degree of hemopoiesis in the bone marrow was evaluated by inspection of the distal femoral and proximal tibial epiphyses. In animals unchallenged by either PG-APS or drugs about 60% of the marrow spaces were occupied by myeloid, erythroid, and megakaryocytic cells, while the remaining 40% were filled with adipose tissue. Three days after the injection of PG-APS into the pouches the fat was reduced to 15%, and on days 6 and 9 it was down to 5 to 10%. Most of the decrease could be attributed to expansion of the number of maturing myeloid cells. CyA completely blocked this hyperplasia and normalized the fat/nonfat ratio (Figure 10). BBE-d,

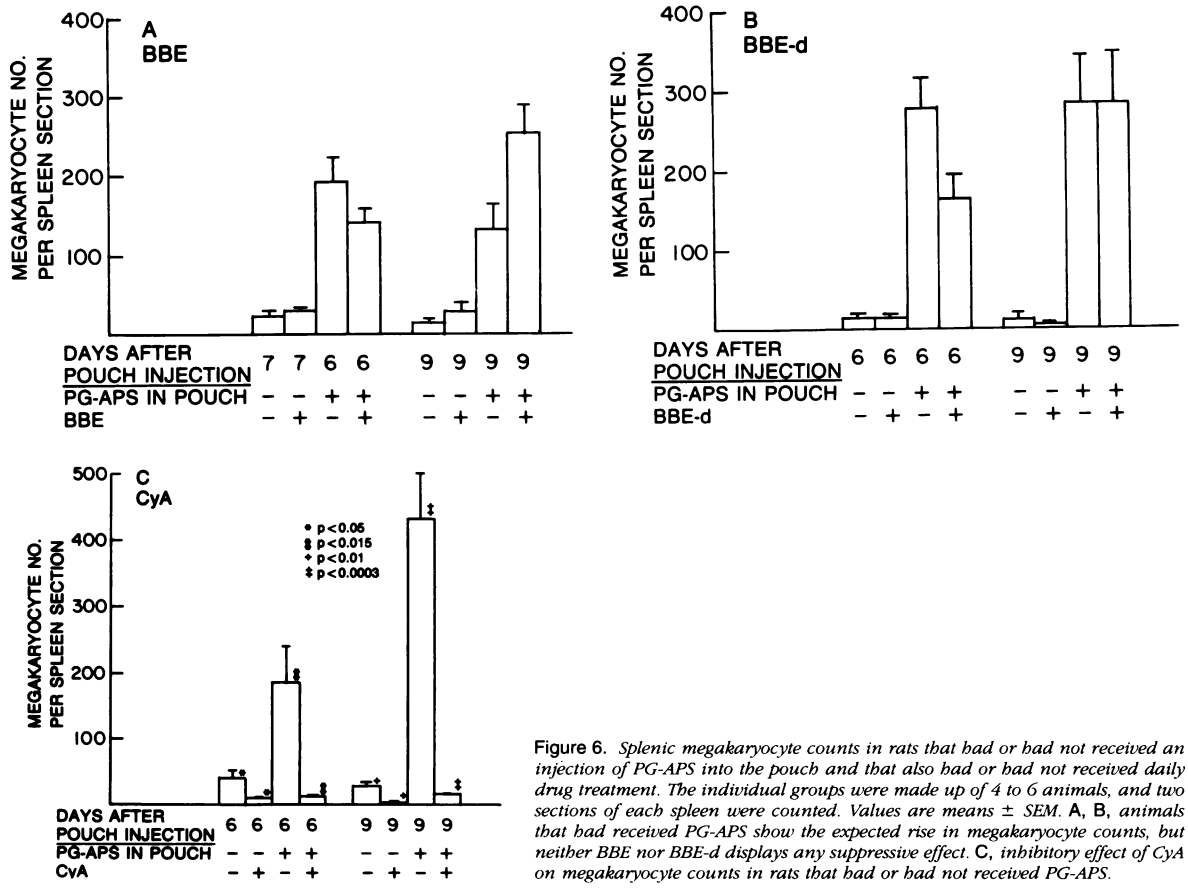

Figure 6. *Splenic megakaryocyte counts in rats that had or had not received an injection of PG-APS into the pouch and that also had or had not received daily drug treatment. The individual groups were made up of 4 to 6 animals, and two sections of each spleen were counted. Values are means ± SEM.* A, B, *animals that had received PG-APS show the expected rise in megakaryocyte counts, but neither BBE nor BBE-d displays any suppressive effect.* C, *inhibitory effect of CyA on megakaryocyte counts in rats that had or had not received PG-APS.*

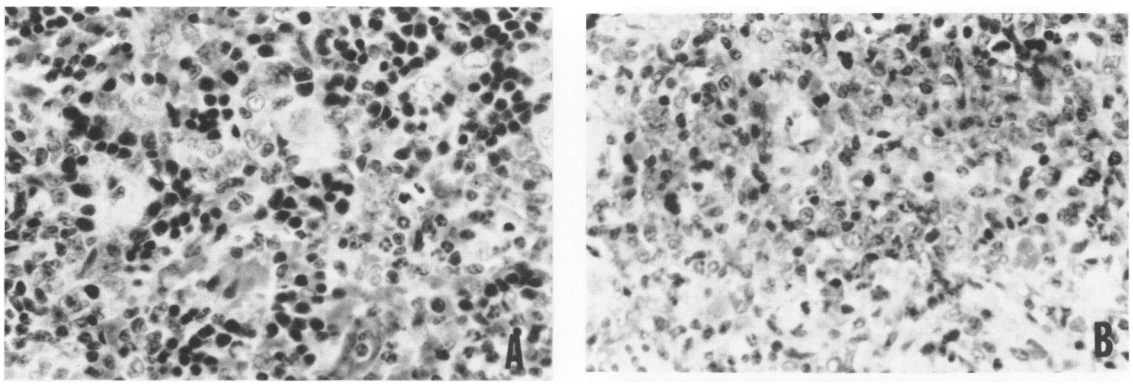

Figure 7. *Splenic red pulp in rats 6 days after injection of PG-APS into the pouch.* **A,** *trilineage hemopoiesis in saline-treated control.* **B,** *absence of megakaryocytic, myeloid and erythroid elements in animal treated with CyA. H&E, ×323.*

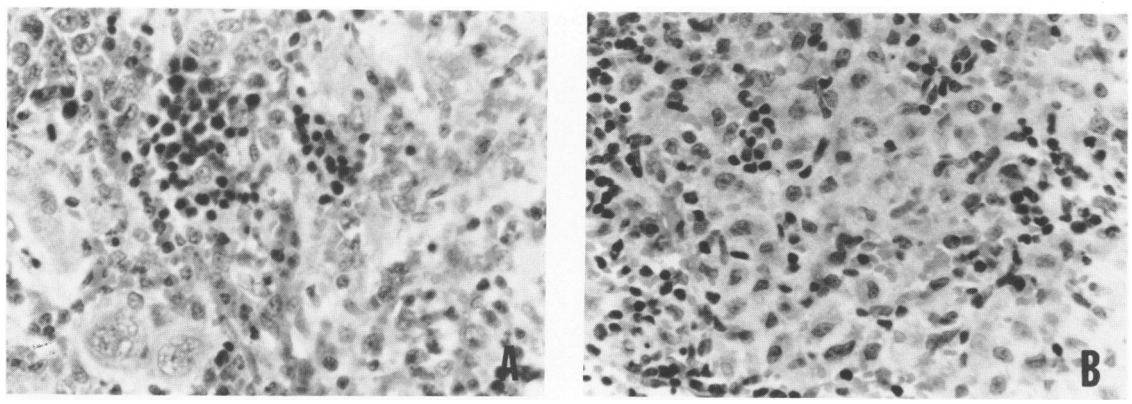

Figure 8. *Splenic hemopoiesis in rats 6 days after injection of PG-APS into the pouch.* **A,** *presence of all three cell lines in saline-treated control.* **B,** *presence of megakaryocytic and myeloid elements, but absence of erythroid precursors, in animal treated with daily injections of BBE. H&E, ×323.*

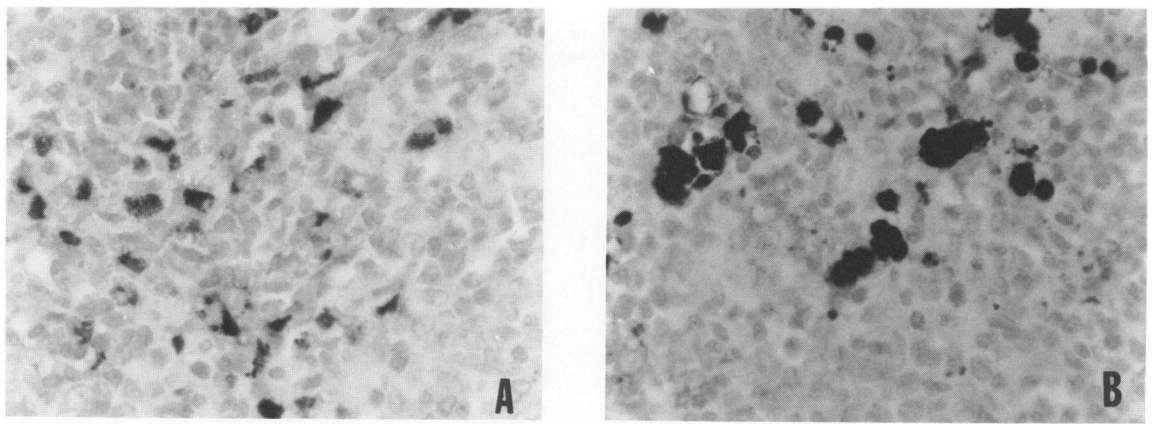

Figure 9. *Iron pigment-containing splenic macrophages in rats that had been given saline, but not PG-APS, into the pouch on day 0 and that had received 9 daily i.v. injections of either saline or BBE.* **A,** *small macrophages in saline control.* **B,** *enlarged macrophages in BBE-treated animal. Turnbull's blue, ×485.*

on the other hand, showed no such beneficial activity, and BBE, as it did in the spleen, greatly decreased the number of red cell precursors. This reduction occurred even if the animals had received the drug only and no PG-APS (Figure 11). Myeloid elements partially filled in for the erythroid components.

To complement the findings in the bone marrow, the impact of the drugs on the circulating white blood cells and on hemoglobin levels was also studied. Data were collected for days 6 and 9 after PG-APS injection. Because of the similarity of the findings at the two time points, however, only the day 9 results are presented (Table 2). For each of

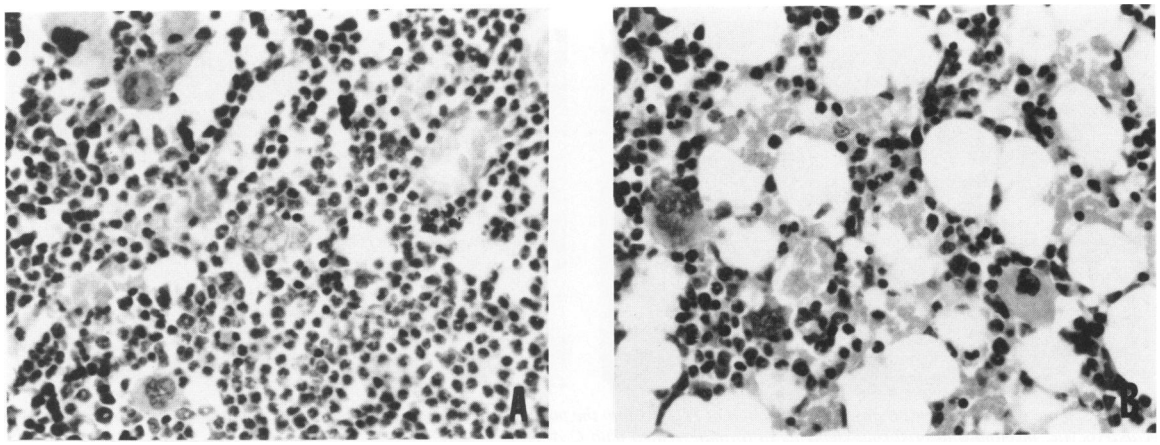

Figure 10. *Bone marrow in distal femoral epiphysis in rats 6 days after injection of PG-APS into pouch.* **A**, *marked hemopoietic hyperplasia with replacement of all fat cells in saline-treated control.* **B**, *normal ratio between hemopoietic elements and fat cells in animal treated with daily i.m. injections of CyA. H&E, ×323.*

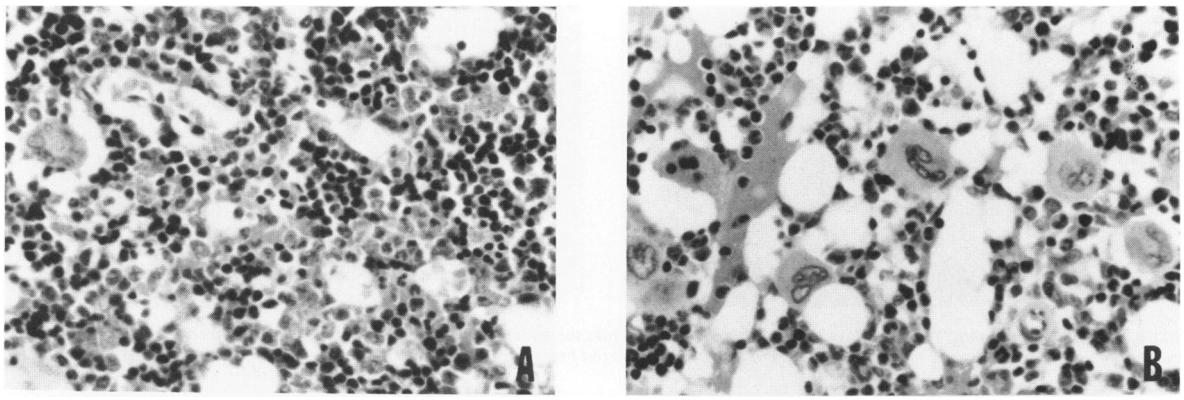

Figure 11. *Distal femoral epiphysis in rats which had an established air pouch, but which had not received PG-APS.* **A**, *normal trilineage hemopoiesis in rat which was given 7 daily injections of vehicle into the pouch.* **B**, *selective absence of red cell precursors in rat which had received 7 daily injections of BBE. H&E, ×323.*

the three compounds there is one group of animals which received PG-APS only and a second group which received PG-APS and the respective drug. As BBE alone had an effect on the values, an additional pair of controls was included with this compound, ie, one group that had received the drug and no PG-APS and another one that had received neither PG-APS nor drug. The latter group can also serve as baseline control for BBE-d and CyA. Two observations can be made on the white blood cell data. First, for all three groups that had been given the drugs the monocyte and lymphocyte counts were higher than in the controls. Second, BBE-d and CyA did not affect the neutrophil number in the circulation, while BBE, with or without PG-APS administration, produced significant neutrophilia.

There was no deleterious effect of BBE-d or CyA on the blood hemoglobin levels. With BBE, however, the suppressive activity on erythropoiesis found its expression in a normocytic anemia with a fall in blood hemoglobin values over the 9-day period from 15.1 to 11.6 g/100 ml.

Discussion

These studies of PG-APS-induced inflammation have expanded our knowledge of the primary events in the air pouch and of the secondary changes in spleen, bone marrow, and circulating blood. As summarized in Table 3 they have also revealed distinctive differences between the modulating effects of CyA, BBE, and BBE-d, and these results make it possible to speculate on possible pathogenetic mechanisms. At the very beginning of the pouch reaction, macrophages and neutrophils are thought to be directed toward PG-APS by chemotaxis. With increasing activation of macrophages, however, secreted interleukin-1 (IL-1) prob-

Table 2. *Influence of BBE, BBE-d, and CyA on the Number of Circulating White Blood Cells*

Drug	Injections		White blood cells (no. per $mm^3 \times 10^{-3}$)*		
	Pouch	i.v. or i.m.	Lymphocytes	Monocytes	Neutrophils
BBE	PG-APS	BBE	11.8 ± 3.3	1.3 ± 0.6	4.3 ± 1.9
	PG-APS	Saline	6.7 ± 0.7	0.2 ± 0.1	0.7 ± 0.2
	Saline	BBE	10.9 ± 0.7	1.7 ± 0.6	3.5 ± 1.5
	Saline	Saline	6.0 ± 1.5	0.3 ± 0.1	0.7 ± 0.2
BBE-d	PG-APS	BBE-d	18.5 ± 2.7	3.2 ± 0.5	2.7 ± 0.6
	PG-APS	Saline	8.8 ± 1.4	0.4 ± 0.1	1.6 ± 0.2
CyA	PG-APS	CyA	10.4 ± 1.7	1.2 ± 0.7	1.6 ± 0.4
	PG-APS	Vehicle	6.8 ± 1.7	0.5 ± 0.2	1.9 ± 0.4

* Blood was collected at the time of sacrifice, 9 days after PG-APS injection.
Injections of BBE and BBE-d were i.v., those of CyA were i.m. n = 5 or 6. Values are means ± SD. i.v., intravenous; i.m., intramuscular.

Table 3. *Modulation of Events in Pouch Model by BBE, BBE-d, and CYA*

Event	BBE	BBE-d	CyA
Inhibition of pouch neutrophilic infiltration	Yes	No	Yes
Enlargement of PG-APS-laden pouch macrophages	Yes	Yes	Slight
Increased size of splenic storage macrophages	Yes	Yes	No
Inhibition of trilineage hemopoietic hyperplasia in spleen	Erythropoiesis only	Partial	Yes
Inhibition of trilineage hemopoietic hyperplasia in bone marrow	Erythropoiesis only	No	Yes
Anemia	Yes	No	No
Blood neutrophilia independent of PG-APS administration	Yes	No	No
Lymphocytosis and monocytosis in PG-APS recipients	Yes	Yes	Yes

ably becomes the driving factor, causing neutrophil extravasation by promoting adhesion to the endothelium and facilitating emigration.[7, 8] CyA, on the other hand, can down-regulate IL-1 production, and this would explain its ability to block the influx of neutrophils into the pouch.[9] Chemotaxis itself, at least under *in vitro* conditions, is not susceptible to inhibition by CyA.[10–12] In addition to the current study, *in vivo* suppression of neutrophil tissue infiltration by CyA has been described in only two situations. In one, the drug reduced the severity of the acute inflammatory cell accumulation that develops during reperfusion of temporarily ischemic intestine.[13] In the other, a beneficial effect of CyA was found in pyoderma gangrenosum, where it promoted the clearing of the mixed acute and chronic inflammatory infiltrate in the skin.[14]

In the PG-APS-dependent arthritis model, BBE treatment prevented the appearance of neutrophils in the bone marrow of the distal tibia, where they would otherwise have been important participants in the osteodestructive process.[3] Furthermore, BBE blocked the characteristic immigration of neutrophils into splenic and hepatic granulomata. Those observations together with the current experience with the air pouch suggest that the antiinflammatory activity of BBE is a more general phenomenon than initially assumed. As with CyA, macrophages probably represent the target of the antiphlogistic effect of BBE, although the actual mechanism of interference is still uncertain. Nevertheless, the interaction of BBE with its binding site must be of high specificity. This is evident from the fact that the overall rather minor change of saturating the central double bond of BBE, and thus turning it into BBE-d, eliminated all antineutrophil activity.

One of the surprising findings in the arthritis studies had been the striking BBE-induced enlargement of PG-APS-laden macrophages in spleen and liver.[3] Now it has become evident that oversized macrophages can occur in tissues other than those two organs and that BBE-d and, to a lesser degree, CyA can also promote the uptake of streptococcal cell wall. Moreover, histological examination of the spleen has shown that excessive storage of material in macrophages is not restricted to PG-APS deposits. Under the influence of BBE and of BBE-d there occurred a redistribution of lipofuscin and iron-containing pigment, some macrophages gaining a large amount and others losing most of their contents. These observations dispel the notion that such stored materials are permanently locked away and would not be subject to further handling. As the drugs obviously alter lysosomal function, it would

be of great interest to know how they would influence reticuloendothelial and parenchymal cells in lysosomal storage diseases, such as sphingolipidoses and mucopolysaccharidoses.

In the pouch model as well as in the arthritis model hemopoietic hyperplasia in the long bones and in the spleen are characteristic features of the disease process. However, there exists a disparity between the two experimental designs in the timing of the histological changes. In the pouch animals proliferation in the two locations developed concurrently, peaked between days 6 and 9, and had subsided by day 17. In the arthritis model, on the other hand, bone marrow hyperplasia was first noted by day 3, while splenic hemopoiesis became obvious 3 days later, by day 6.[2] The involvement of the long bones reached a prolonged high plateau by day 6, and the splenic changes again lagged 3 days behind, achieving a high plateau by day 9. The sequence of events in the two experimental systems was probably a reflection of the pattern of release of stimulating mediators. In the pouch model there most likely was a single pulselike discharge of the agents responsible. In the arthritis model it appears that the initial stimulus might have originated in the bone marrow macrophages, which are known to acquire PG-APS.[15] The splenic hemopoiesis, however, and possibly also the persistent hyperplasia in the bone marrow, are best attributed to the release of cytokines from the increasingly more massive splenic and hepatic granulomata. Those granulomata are already known to be involved in the synthesis and secretion of IL-1, IL-2, and IL-3.[16] Participation of cytokines is supported by the experience of other investigators who have shown that interleukins-1, -2, -3, and -6 are elevated in the hyperplastic bone marrow of rats with collagen-induced arthritis[17, 18] and that interleukins-1 and -6 are increased in the marrow of animals with yet another type of arthritis, ie, adjuvant-induced arthritis.[18] From these data it is probably safe to conclude that the antihemopoietic properties of CyA are based on the reduction of cytokine activity. Although this interference is beneficial to the animals in the PG-APS-induced disease model, one wonders what role cytokines might play in the regulation of normal hemopoiesis and if treatment with CyA might cause or intensify anemia in humans.

At this point there is no satisfactory explanation for the selective inhibition of erythropoiesis by BBE. Conceivably, it could be a toxic effect, it could result from competition for growth factor receptors, or it could be due to inhibition of the function of the nurturing macrophages of the erythroblastic islands.[19–22] It is also uncertain at this point if the neutrophilia caused by BBE is connected to the anti-erythroid activity.

Additional studies will be necessary to determine if the lymphocytosis and monocytosis associated with all three drugs are due to increased cell mobilization or to prolonged intravascular retention of the cells.

In summary, streptococcal cell wall-induced inflammation in the rat air pouch has proved a valuable system for the testing of the antiphlogistic effects of macrophage-directed compounds. As demonstrated above, size of the macrophages, number of infiltrating neutrophils, and degree of hemopoietic stimulation in spleen and bone marrow are some of the parameters that can be explored. The model has revealed new interventional properties of CyA, and its use is recommended in future evaluation of CyA-like agents.

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