

Studies on the adjuvant action of beryllium

IV. THE PREPARATION OF BERYLLIUM CONTAINING MACROMOLECULES THAT INDUCE IMMUNOBLAST RESPONSES *IN VIVO*

J. G. HALL *Experimental Unit, Section of Medicine, Institute of Cancer Research, Royal Marsden Hospital, Sutton, Surrey*

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SUMMARY

Solutions of BeSO₄ were added to soluble macromolecules and the mixtures brought to neutrality. Under appropriate conditions much of the beryllium did not precipitate out as insoluble hydroxides but became attached to naturally occurring sulphated proteoglycans and polysaccharides (e.g. heparin, chondroitin sulphate, fucoidan, etc.) and to synthetic, sulphonated aromatic dyestuffs (e.g. trypan, Evans and Coomassie blues, Suramin, etc.). At physiological conditions of pH and ionic strength, these addition compounds were relatively stable and did not dissociate during dialysis. When such materials were injected s.c. into sheep (in doses containing about 50 µg Be), rapid lymphoproliferative responses took place in the regional nodes so that immunoblasts appeared in the efferent lymph in numbers that exceeded those provoked by powerful conventional antigenic stimuli. Significant amounts of the injected materials passed through the nodes and, in intact animals, became systematized. A method was devised for attaching beryllium to particulate carriers, such as lymphocytes or red cells that had been fixed with glutaraldehyde. The injection of suspensions of such materials also provoked vigorous immunoblastic responses, but the particulate materials did not pass beyond the regional node. In the doses usually used, none of these materials was grossly toxic and they may be suitable for consideration for development into a new type of immunostimulator cum adjuvant.

INTRODUCTION

The experiments reported in the previous paper (p. 341) showed that the adjuvant properties of beryllium were not confined to the damaging effects of insoluble microcrystalline salts of beryllium, but could be expressed by soluble and relatively non-toxic complexes of beryllium and plasma proteins. However, the exact nature of these complexes was quite obscure. Because of the potential variety and instability of the complexes generated *in vivo* by the interactions between beryllium and a large range of different proteins, it seemed that the direct investigation of such material would be very difficult. Instead, it seemed more profitable to react beryllium with single, defined molecular entities *in vitro*, and then to test the resulting addition compounds (adducts) for activity *in vivo*.

One reason for doing this was the hope that new and usable adjuvants and immunostimulators might be developed. Accordingly, as the work progressed, potential carriers for beryllium were chosen, where possible, from compounds that were easy to sterilize and store or that had been administered already to animals or man, either as adjuvants or for other therapeutic or diagnostic purposes.

Correspondence: Professor J. G. Hall, Experimental Unit, Section of Medicine, Block X, Institute of Cancer Research, Royal Marsden Hospital, Downs Road, Sutton, Surrey SM2 5PX, U.K.

MATERIALS AND METHODS

General procedure for the addition of beryllium to carrier molecules

Because of the capricious behaviour of beryllium, it was necessary to monitor all stages of the process by incorporating sufficient ⁷Be (as ⁷BeCl₂, carrier-free; Radiochemical Centre, Amersham, Bucks) into the system to give a convenient counting rate so that the radioactivity could be assayed easily and quickly at appropriate times. This was particularly necessary during the use of strongly coloured materials that masked the occurrence of turbidity or even frank precipitation.

A stock solution of BeSO₄ · 4H₂O (Analar Grade, Sigma Chemical Co. Ltd, Poole, Dorset) was made by dissolving 1.0 g in 100 ml of distilled water. Usually, 0.1 ml (containing approximately 50 µg of Be) was taken and mixed with the small tracer dose of ⁷BeCl₂. The mixture was then added to 2.0 ml of a 1% solution in distilled water of the carrier molecule under test. The acidity of the BeSO₄ was neutralized by the drop-wise addition of N/10 NaOH, and the mixture was transferred quantitatively to a dialysis sack that was immediately sealed, rinsed and wiped dry. It was convenient to place the entire sack in the well of a γ-spectrometer (model '1100', Wilj Electronics, Ashford, Kent) to establish the initial counting rate. The dialysis sack was placed first in running tap water for 5 hr and then in a

1000 volumes of constantly stirred distilled water (at room temperature) for a further 15–20 hr. At convenient intervals the radioactivity of the dialysis sack was re-assayed. When the preparation was 'successful', any free beryllium had passed out of the dialysis sack in the first few hours, and after this the amount of retained radioactivity decreased only slightly. At the end of the dialysis period the contents of the sack were withdrawn and brought to physiological tonicity by the addition of a 1/10th volume of 1.5 M NaCl. The material was then centrifuged at 1200 g and the supernate assayed for radioactivity so that the amount of beryllium remaining attached to the soluble carrier could be calculated. With all but the most 'avid' carriers this rarely exceeded 75% of the initial amount.

Larger batches were prepared in the same way by simply scaling up the process but, of course, it was only possible to assay the radioactivity at the beginning and end of the procedure.

Usually, materials were submitted to bioassay as soon as they had been prepared. When this was not possible they were stored at 4° until required, but were always subjected to a further centrifugation and radioassay immediately before use.

Details of minor departures from this procedure will be described in the Results.

The main problem in these preparations was the possibility of the beryllium precipitating out as an insoluble hydroxide, but the presence of a sufficiently avid carrier usually prevented this. However, there were other reasons for failure. Prolonged dialysis against tap water sometimes caused precipitation, presumably because the small amounts of divalent metallic cations present interfered with the interaction between beryllium and the carrier. Similarly, the addition to the beryllium-carrier adducts of phosphate-buffered saline (PBS), instead of ordinary saline, sometimes brought about a substantial precipitate of insoluble beryllium phosphate.

In all cases the bulk of the beryllium bound to the carriers could be removed by elution with acid (pH 3).

Carrier materials

Bovine serum albumen (BSA; crystalline, globulin free), bovine heparin, chondroitin sulphate, fucoidan and gelatin (porcine) were obtained from the Sigma Chemical Co. Pharmaceutical preparations of heparin ('Pularin', Duncan and Flockhart Co. Ltd, London and 'Monoparin', Weddel Pharmaceuticals Ltd., Wrexham, Clwyd) were used also.

Trypan blue was obtained as a sterile 0.5% solution from Flow Laboratories (Irvine, Renfrewshire). Coomassie blue (brilliant blue R), and Evans blue were obtained from Sigma Ltd. 'Suramin' was obtained from Bayer, West Sussex (Haywards Heath).

Solutions of the dyestuffs often contained aggregated material, and it was found to be essential to remove these by centrifugation for 10 min at 1200 g immediately before adding the solution of BeSO₄.

The addition of beryllium to lymphocytes and red cells

Originally, this method was devised to couple beryllium to living, unfixed lymphocytes and rather mild conditions had to be used. The general procedure was as follows. Approximately 5×10^8 washed lymphocytes, obtained from the lymph of sheep, were suspended in 50 ml of 0.15 M sodium acetate, to which 0.1 ml of 1.0 ml of 1.0% BeSO₄.4H₂O and a tracer dose of ⁷Be had

been added. This amount of BeSO₄ was usually sufficient to bring the pH of the (alkaline) acetate solution to near physiological pH without causing any precipitation. The cells were allowed to stand in this mixture, which contained approximately 1 µg Be per ml, for 15 min at room temperature before being deposited by centrifugation, washed once and resuspended in 5 ml isologous lymph plasma, prior to bioassay by s.c. injection into a sheep.

Although living lymphocytes occasionally survived this treatment, they were sometimes obviously damaged and underwent agglutination and lysis. It was more convenient, therefore, to fix the lymphocytes with 1% glutaraldehyde in PBS at pH 7.4, for 5 min at room temperature. The fixed cells were then washed twice in distilled water and treated as above. At the end of the process the cells were found to have retained about 60% of the beryllium initially present.

Sheep red cells were found to be too fragile to be treated in the unfixed state but, after fixation, they were treated as above in batches of approximately 5×10^9 .

Bioassay of beryllium-carrier adducts

All the various preparations either were or behaved like macromolecules, and so were transported by the lymphatic system after s.c. injection. Their biological effect was measured in terms of the numbers of blast cells that appeared in the efferent lymph from individual lymph nodes in unanaesthetized sheep after a measured dose had been injected into the drainage area of the node.

Sheep, either wethers or ewes, weighing approximately 30 kg, were prepared surgically so that they had an indwelling cannula in the efferent duct of either a popliteal (Hall & Morris, 1962) or prefemoral (Hall, 1967) lymph node. The lymph was collected quantitatively into sterile, heparinized polythene bottles that were changed at appropriate intervals, and never less than twice per day. The volume and lymphocyte count of each collection were measured and the percentage of immunoblasts present was determined by counting a total of at least 500 living cells in whole lymph, using phase-contrast optics. Also, the ultrastructure of the blast cells was investigated by standard electron microscopic techniques. The immunoglobulin content of the blasts was monitored by immunoperoxidase staining of methanol-fixed cell films, and by immuno-diffusion and immuno-electrophoretic studies of detergent extracts of washed lymph cells (Hall, Hopkins & Orlans, 1977; Hall, Hopkins & Reynolds, 1980).

The dose of material to be injected was mixed with 5 ml of isologous lymph plasma and, where colourless materials were being used, the injection was coloured with lymphography dye. After s.c. injection, the rate of appearance of dye in the efferent lymph of the regional node confirmed that the material had been injected in the right place and had reached the node. Occasionally this did not happen and the experiment was aborted.

Under normal conditions the lymph from the popliteal and prefemoral nodes is populated almost exclusively by normal small lymphocytes, with only 1–2% of blast cells. The appearance of substantial numbers of such cells is usually the result of antigenic stimulation and, in primary immune responses, they reach a peak value about 90–100 hr after the antigen has been administered (Hall, 1971). When beryllium is used as the stimulus the response is more rapid (Hall, 1984) so that the peak efflux of immunoblasts occurs within 70–80 hr. For the purposes

of this paper, the percentage of immunoblasts in lymph collected between 70 and 75 hr after the injection of the test material was taken as the prime measure of its effect.

None of the carrier materials caused any significant immunoblast response when injected by themselves, and there is abundant experimental experience with heparin, Evans blue, trypan blue and Suramin which attests to this. BSA is potentially immunogenic, but in practice caused little significant reaction in sheep unless the animal had been hyper-immunized previously with the material. Similarly, fucoidan has been shown to be relatively non-immunogenic in the sheep (Hall, 1986), and the same can be said of gelatin (Hall & Morris, 1965).

Distribution in vivo of beryllium-carrier adducts

Because ^7Be was incorporated always in the various, non-particulate preparations, it was possible to monitor the distribution of the injected material in small animals. One-millilitre doses (usually containing over 60,000 c.p.m. of radioactivity) were injected into the jugular veins of 250 g, male Wistar rats (National Institute for Medical Research, London) that had been anaesthetized with ether. The animals were allowed to recover and were kept overnight before being killed so that selected organs could be assayed for their γ -emissions.

RESULTS

Preliminary experiments

At first, attempts were made to couple beryllium to whole plasma by the methods described. Although the actual conjugation was successful, it was found that different preparations produced very different responses *in vivo*, even though they contained similar amounts of beryllium. In order to try and produce a more uniform and easily sterilizable material, gelatin was tried; it proved to be a poor carrier, nearly 75% of the beryllium being lost during dialysis. Although the final preparation had unequivocal activity *in vivo* it was unstable; the beryllium tended to precipitate out spontaneously and was precipitated always on the addition of PBS. BSA was a more satisfactory carrier. It retained 70–75% of the added dose of beryllium and was relatively stable and compatible with PBS. It produced significant immunoblast responses *in vivo* (Table 1) but because of its potential immunogenicity and perishability it was not investigated further.

Sulphated polysaccharides as carriers for beryllium

A consideration of the composition of whole plasma used in the preliminary experiments suggested that the role of heparin, which was used as the anti-coagulant, should be investigated.

Heparin was found to be a useful carrier of beryllium, even when used at the relatively low, pharmaceutical concentration of approximately 0.7% (i.e. 7 mg/ml equivalent usually to about 1000 IU/ml). Its behaviour during dialysis is shown in Fig. 1; it usually retained some 75% of the starting dose of beryllium and yielded perfectly clear solutions, whose radioactivity was not decreased by vigorous centrifugation. These solutions remained stable at 4° for several weeks. Chondroitin sulphate behaved in a similar manner to heparin. Fucoidan, (a sulphated poly-fucose) was more avid than either and always retained virtually the whole dose of beryllium, as shown in Fig. 1. Indeed, the

Table 1. The percentages of immunoblasts in lymph, efferent from the popliteal or prefemoral nodes of sheep, collected between 70 and 75 hr after the nodes had been stimulated by the s.c. injection of one of a variety of Be-carrier adducts. Each percentage shown refers to a different animal preparation

Carrier	Approx. content of beryllium (μg)	% immunoblasts in lymph 70–75 hr after injection
25 mg bovine serum albumen	35	25,28,39
14 mg heparin	30	33,32,46
20 mg chondroitin sulphate	30	37,28
20 mg fucoidan	50	44,35,37,39
10 mg Trypan blue	30	34,40,38,29
20 mg Evans blue	35	49,40,35
20 mg Coomassie blue	30	26,32
20 mg Suramin	15	30,52,28
5×10^8 fixed lymphocytes	30	38,40,30,42,57
5×10^8 fixed lymphocytes	3	26,22
5×10^9 fixed SRBC	35	48,61,73,56,35

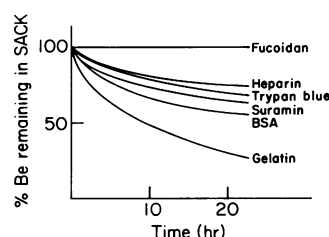


Figure 1. Specimen curves showing the decreases in radioactivity, during dialysis under neutral conditions at room temperature, of various Be-carrier mixtures labelled with ^7Be . At the start of the dialysis each 2-ml system contained approximately 50 μg Be and 10–20 mg of the carrier material. It can be seen that fucoidan retained all of the beryllium, while gelatin lost most of it. The other carriers all retained between 60% and 75%.

standard 1% solution of fucoidan could bind up to 400 μg of beryllium per ml (over 15 times the standard dose) and still not show any loss of activity after prolonged dialysis and final centrifugation.

All these materials were capable of provoking vigorous immunoblast responses in the lymph of sheep (Table 1) at doses containing from 15 to 50 μg of beryllium. None of them caused palpable induration or granulomata at the injection site, though the injection of heparin occasionally elicited a small local haematoma. None of the materials was retained quantitatively by the local node. It was always possible to detect some radioactivity in the efferent lymph in the 24 hr after injection. The relatively small amounts of radioactivity rendered accurate quantification difficult, as did the fact that some of the injected material must have drained to other nodes. Usually some 20–30% of the injected radioactivity appeared in the efferent lymph and some remained at the injection site under the skin and in the regional node.

In the doses used here, none of the carriers was capable in its native state of inducing any significant immunoblast response in the efferent lymph.

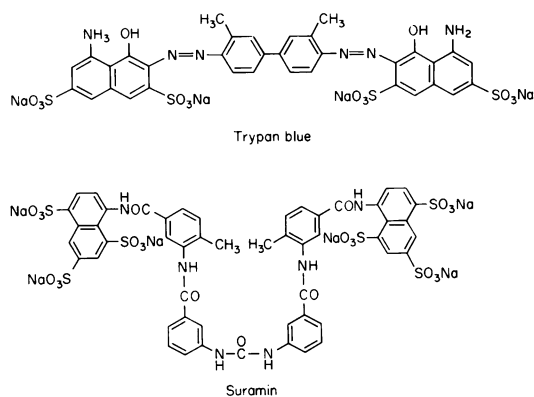


Figure 2. Structural formulae of trypan blue and suramin to show the general features of this class of chemicals: note the frequent sulphonic acid residues.

Sulphonated dyestuffs as carriers for beryllium

The success of the sulphated polysaccharides, etc., as carriers suggested that the charged sulphate groups might have been responsible for binding the beryllium ion, and it seemed worth testing this by using a different type of molecule. Dyes of the trypan blue class have several sulphonic acid residues in the form of sodium salts, although the actual molecular weight of such dyes is usually under 1000, they behave operationally as macromolecules and are, for the most part, retained within standard dialysis tubing. All these dyes have a generally similar structure, and specimen formulae are shown in Fig. 2. All the three dyes tested, i.e. trypan, Evans and Coomassie blues, behaved similarly during dialysis (Fig. 1) and retained about 75% of the starting dose of beryllium. All were active *in vivo* and provoked strong immunoblastic responses (Table 1).

Suramin is a colourless compound of similar general composition to the above dyes, and has been used as a drug against trypanosomes and filaria. It behaved similarly to the dyes during dialysis but some turbidity generally developed so that up to 50% of the residual radioactivity could be removed by centrifugation. The clear supernatant solutions, however, did exhibit a substantial effect *in vivo* (Table 1).

It was possible with the coloured dyes to see that substantial amounts of the injected material passed through the regional node and entered the efferent lymph. A count of the radioactivity in the lymph plasma showed that this could amount to 30% of the injected dose. Suramin behaved similarly.

Again, none of the dyestuffs caused any significant immunoblast response unless they were complexed with beryllium.

Distribution of beryllium-carrier adducts *in vivo*

Because none of the materials tested were retained quantitatively by a single node, the presumption must be that, in an intact animal, some of the injected dose would ultimately gain the blood stream. In order to find out how such materials were distributed by the systemic circulation, 1.0-ml doses were injected i.v. into rats, whose tissues were assayed for radioactivity 20 hr later. The results are shown in Table 2. As might have been predicted, detectable amounts of radioactivity were associated with the organs of the reticulo-endothelial system, and the liver, being the largest, received the most. However, some

Table 2. The distribution of radioactivity in the organs of rats 20 hr after the i.v. injection of standard Be-carrier adducts labelled with ^7Be . A dose containing 500–1000 c.p.m. was injected into each rat and the activity detected later in the tissues was expressed as a percentage of this; the percentages were corrected to the nearest whole number, and values less than 0.6 were scored as zero. Each set of figures refers to a single rat

	Carrier							
	Acetate buffer	Fucoidan	Heparin	Trypan blue	Evans blue	Suramin		
Liver	40,45	35,30,30,39,36	20,21	31,20,18	26,27	14,20		
Spleen	2, 3	7, 8,12, 8, 5	5, 6	5, 7, 8	8, 7	6, 7		
Lungs	0, 0	2, 1, 1, 0, 0	0, 0	0, 1, 0	1, 1	0, 0		
Small gut	0, 0	0, 0, 1, 2, 2	1, 2	0, 1, 0	1, 1	1, 1		
Kidneys	1, 1	2, 3, 4, 1, 1	2, 1	1, 2, 1	1, 1	1, 1		
Femoral Bone marrow	0, 0	2, 2, 1, 1, 1	1, 1	4, 3, 3	3, 3	1, 2		

Be-trypan blue, and Be-Evans blue seemed particularly able to evade quantitative retention by the liver and spleen so that significant amounts were detectable in the bone marrow. A few of the rats were kept for up to 7 days after the injection and, although the total amount of radioactivity had declined slightly, the pattern of distribution remained unaltered.

Particulate carriers of beryllium

Because the soluble carriers of beryllium had the general property of escaping quantitative retention by the regional node, it was considered likely that particulate carriers, which tend to be retained more effectively (Hall *et al.* 1967), might be even more effective in provoking immunoblastic responses to beryllium in the regional nodes. Lymphocytes and red cells were chosen as particulate carriers because they could be obtained easily in quantity and represented a convenient source of biodegradable 'microspheres'. In this study, only autochthonous cells were used so that complications arising from the immunogenicity of allogeneic cells could be excluded, although, in fact, later experiments have shown that allogeneic, fixed cells provoke the same responses as isogeneic ones.

The meagre buffering capacity of 0.15 M acetate limited the concentration of beryllium to which the lymphocytes could be exposed and, as Fig. 3 shows, the capacity of lymphocytes for beryllium probably was not saturated. Nonetheless, the treated cells provoked vigorous immunoblastic reactions (Table 1) and some of these were the largest yet recorded. Even cells treated with one-tenth of the usual concentration of beryllium were able to induce substantial reactions. The use of ^7Be -labelled lymphocytes showed that none of the injected dose succeeded in passing into the efferent lymph. However, as usual with lymphocytes that have been injected s.c., much of the injected dose remained at the injection site and it is likely that only a minority actually reached the node. Thus, in these experiments the amount of beryllium that actually induced the responses may have been quite small.

Fixed red cells, too, were effective in binding beryllium and in provoking immunoblastic responses, but their content of

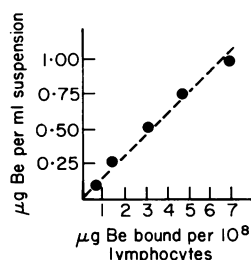


Figure 3. The relationship between the concentration of beryllium in the suspending medium (0.15 M sodium acetate) and the amount of beryllium that was bound to the lymphocytes after they had been suspended for 15 min at room temperature and then washed. For practical purposes the relationship was linear. It seems likely, therefore, that the capacity of the cells for beryllium was not saturated, even at the highest concentration that the system could accommodate. The results illustrated were obtained from an experiment in which ⁷Be was used but essentially similar results were obtained by measuring the bound beryllium directly, by atomic absorbance spectroscopy.

fixed haemoglobin resulted sometimes in permanent discoloration at the sites of injection.

It was not feasible as a routine to determine the *in vivo* distribution of fixed beryllium-treated cells after *i.v.* injection into rats. The treated cells were retained quantitatively in the lungs and often caused fatal embolism.

Nature of immunoblasts produced in response to beryllium-carrier adducts

The immunoblasts produced in response to beryllium-carrier adducts were identical in morphological and ultrastructural terms with those produced by conventional antigens (Hall *et al.*, 1967). Similarly, the immunoglobulin content of the blast cells was made up principally of IgG1, with detectable IgG2 and IgM but no IgA, and was thus similar to that of immunoblasts produced in response to bacteria viruses, homografts and skin-sensitizing chemicals (Hall, 1971; Hall *et al.*, 1977, 1980). As in previous studies of reactions to beryllium (Hall, 1984), no evidence could be found that either humoral or cell-bound immunoglobulin was capable of any immunologically specific interaction with bound or free beryllium.

A substantial minority of the immunoblasts was completely negative in immuno-cytological tests for intra-cytoplasmic immunoglobulin and must be presumed to have been T cells.

Thus in all particulars that could be studied, the immunoblasts produced in response to beryllium-carrier adducts were indistinguishable in cytological terms from those produced during responses to conventional antigens.

High dose effects

Most of the soluble carriers used were, for practical purposes, saturated with beryllium, and the dose of beryllium could be increased only by increasing the volume of material injected. However, fucoidan (unfortunately, the least well characterized of the carriers studied) was an exception to this and its high avidity for beryllium made possible the preparation of fully soluble material containing some 20 times the usual content of beryllium. In this way it was possible to give single doses containing 1 mg of beryllium *s.c.* to each of two sheep. When this

was done, the responses in the efferent lymph initially resembled those observed in response to large doses of insoluble Be(OH)₂ (Hall, 1984). Many dead cells, some granulocytes and dendritic macrophages appeared in the lymph but, before the immunoblast response was established the flow of lymph dwindled and ceased. Under these circumstances there was nothing to be lost by killing the sheep and removing the lymph nodes. It was found that the regional nodes, which weigh normally about 1 g, were grossly enlarged; one weighed 8 g, the other 12 g. Some of this enormous increase could be accounted for by inflammatory oedema but histological examination showed the granulocytic infiltrate to be relatively sparse; the striking feature was widespread sinus histiocytosis.

Doses of 1 ml of fucoidan containing approximately 375 µg of beryllium were injected *i.v.* into each of three rats. The animals did not die but were obviously not very well and were killed a week later. The only obvious abnormality was enlargement of the spleen, but histological examination of the liver showed small areas of periportal necrosis in all lobes (Witschi, 1971).

DISCUSSION

The biological significance of the immunoblast responses provoked in efferent lymph by the local injection of beryllium-carrier adducts can be judged best by comparing them with those provoked by conventional antigens. Even the most powerful antigenic stimuli (e.g. swine influenza virus; Smith & Morris, 1970) given to specifically pre-immunized sheep usually provoke the appearance in the lymph of no more than 40% of immunoblasts. Thus, when percentages of this order, and sometimes more, occur within 75 hr of a single injection of apparently non-antigenic material it is hard not to be impressed. This impression was strengthened by the fact that the total doses of beryllium were in the microgramme range, much less than the milligramme doses of insoluble, inorganic beryllium needed to achieve the same effect (Hall, 1984), and that overt toxicity seemed negligible.

The mechanisms involved are unclear. The beryllium was apparently bonded ionically to the carriers, and the possibility that, *in vivo*, it became detached in the extracellular phase and formed insoluble salts that were phagocytosed directly cannot be excluded. In any event, it is more than likely, because of the nature of the carriers and the known behaviour of beryllium (Hall, 1984), that an interaction between macrophages in the lymph nodes and the beryllium-carrier adduct was the primary event. Certainly, the adjuvant action of beryllium has been attributed to increased antigen presentation by affected macrophages (Behbehani, Beller & Unanue, 1985). Nonetheless, a direct interaction between beryllium and lymphocytes cannot be discounted. Skilleter & Price (1984) have shown that this can occur, and drew attention to the capacity of beryllium to bind specifically to the acidic, non-histone nuclear proteins (Parker & Stevens, 1979) which regulate cell division. Irrespective of mechanism, the net effect in sheep was a non-specific, polyclonal, lymphoid blastogenesis, the product of which seemed identical to that which characterizes genuine immune responses *in vivo* both B and T lymphocytes being driven to undergo transformation into blast cells. During the course of this work monoclonal antibodies specific for the T cells of sheep became available (Beya *et al.*, 1986), and by using them it was possible to

show that some 15–20% of the immunoblasts produced in response to beryllium were T cells. The involvement of T cells accords with the results of the investigation of cellular reactions to beryllium in other species (Maceira *et al.*, 1984).

The large scale generation of immunoblasts, *in vivo*, in response to relatively simple, non-antigenic chemicals in non-toxic doses has not been recorded often. The long-term administration of diphenylhydantoin for the treatment of epilepsy in man occasionally induces an immunoblastic lymphadenopathy (Lapes, Vivaqua & Antoniadis, 1976). Similar changes, which resulted sometimes in a GVHD-like syndrome, have been reported in rodents (Gleichmann, Pals & Radeszkiewicz, 1983), but large (200 mg), single doses of sodium diphenylhydantoinate injected s.c. into sheep caused only minor (10%) and late (100 hr) immunoblast responses in lymph efferent from the regional nodes.

Whether the lympho-proliferative activity of the beryllium-carrier adducts is related in any way to the general ability of beryllium to act as an immunological adjuvant is a question that can be answered only by an exhaustive screening programme utilizing small animals. This has yet to be done. The *i.v.* injection of such materials into rats resulted, within a few days, in relative and absolute monocytosis in the blood, which may reflect the entry of the injected material into the bone marrow. However, it should be noted that carriers like trypan blue (Kripke *et al.*, 1977), Evans blue (Anderson & Crowle, 1981), Suramin (Pesanti, 1978; Brandley, Lagrange & Hurtrel, 1986) and chondroitin sulphate (Rosen *et al.*, 1987) have some immunological effects in their native state. These 'soluble' carriers were chosen because of their known ability to become associated with plasma proteins and to circulate for some time before being sequestered by the reticulo-endothelial system. It was thought that this property might be important for adjuvant activity aimed at the induction of a comprehensive, systemic immune response. Conversely, the particulate carriers were designed to limit the initial stimulus to a regional node. However, neither the soluble nor the particulate carriers described above must be regarded in any sense as definitive or final; they are only examples, and merely represent familiar materials that were readily available. Many superior carriers may exist. Although not described in the results, dextran sulphate, carrageenan and tragacanth have been used successfully as a soluble carriers. Synthetic microspheres of agarose, albumen and starch or suspensions of killed bacteria have been found to be just as good as particulate carriers as fixed lymphocytes and red cells.

Although the exposure under industrial conditions of the respiratory tract to large doses of beryllium can produce the pulmonary and other manifestations of 'berylliosis', there is no evidence that the small doses usually used in the present study have any significant, toxic or sensitizing effects, but the possibility of beryllium 'hypersensitivity' must be considered. There is considerable doubt whether hypersensitivity to beryllium, in the true immunological sense, really exists, though there is a mass of conflicting data on alleged cutaneous hypersensitivity in patients with berylliosis (Tepper, 1980). In most animal studies the genotype of the recipient is more important than a history of prior exposure in determining the reaction of an individual to beryllium (Barna *et al.*, 1984a, b). Certainly, in sheep which had been observed over a period of years, repeated doses of beryllium-containing materials provoked the same reaction as the primary dose, and no hypersensitivity reactions

were observed. Similarly, it was impossible to demonstrate the induction of immunoglobulins or other macromolecules that reacted specifically with beryllium or beryllium-carrier conjugates.

Finally, although the study was pursued in the context of immunology it is known that beryllium inhibits many enzymes (Reiner, 1971), so it could be used to intervene in a number of biochemical situations if appropriate carriers could be used to guide it to the right place and prevent it reacting prematurely with plasma proteins and phagocytic cells.

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