Influence of Bacille Calmette-Guérin Infection on Polyoma in Hamsters and Mice

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Summary

In hamsters inoculated with tubercle bacilli of the Calmette-Guérin strain (BCG) a week after birth and later with polyoma virus, the incidence of malignant changes was reduced and survival was considerably prolonged; hemagglutination-inhibiting, complement-fixing, and neutralizing antibodies against the virus were augmented. However, in controls given polyoma virus but no BCG, animals with higher titers of antibodies died earlier and had more neoplastic lesions than those with low titers. This suggests the presence of other types of antibodies, which would also be stimulated by BCG and would be responsible for protection against neoplasia.

In mice inoculated with the virus at birth and with BCG later, the incidence of tumors was not changed, but other results were similar to those seen in hamsters (prolonged life, increased antibodies), although less significant.

Introduction

Tubercle bacilli of the Calmette-Guérin strain (BCG) have been shown to exert protective effects against some transplanted tumors (1, 7, 11–13, 22) as well as against spontaneous leukemia (11, 12) and mammary carcinoma (12). Preliminary experiments indicated that the development of polyoma tumors was delayed and life was prolonged in hamsters infected with BCG (11). The present work was undertaken to investigate further the antagonistic influence of BCG on polyoma in hamsters and to extend this study to mice. The incidence of pathologic changes and survival time were observed in animals given the polyoma virus with or without BCG. As immunologic responses may be an instrument through which the protective action is effected, antibodies against polyoma were evaluated in the serum of the animals, in an effort to analyze the mechanism of protection.

Materials and Methods

ANIMALS. Hamsters were random-bred animals from the colony of golden hamsters established in this laboratory in 1961, the progeny of 2 pregnant females bought from Bio-Research Consultants (Cambridge, Mass.). The animals are usually discarded after 1 year of age, which corresponds to the duration of the present experiment. In these hamsters, the incidence of spontaneous tumors is very low, i.e., less than 1%.

Mice were C3Hf animals of our inbred substrain, which has been derived, by brother-sister mating, from a pair of breeders obtained from L. Gross in 1955; these belonged to an inbred line, C3Hf/Bi, in which the milk factor had been eliminated by foster nursing, as described by Gross (4). On the basis of “Standardized Nomenclature” (21), the full name of the present substrain is C3Hf/BiGxLe. The incidence of spontaneous tumors of the liver and the digestive tract in these mice is around 7% and that of leukemia is 2%; other tumors are rare, with an incidence of less than 1%; mammary carcinomas were never observed.

POLYOMA. The strain of virus used was isolated in 1961, in C3Hf mouse embryo tissue culture from a filtrate of pooled tissues from 3 AK mice, and has been maintained since then by passages in similar cultures, with intervening periods of storage at —60°C. The identity of this virus as polyoma virus was repeatedly established by its tumor-inducing activity in hamsters and mice, cytopathic effects in mouse tissue culture, and hemagglutination, as well as by its neutralization by a specific antiserum (kindly supplied by A. A. Axelrad and Rose Sheinin of the Ontario Cancer Institute).

TISSUE CULTURES. Mouse embryo cultures were used to obtain viruses for inoculations of animals, and for hemagglutination inhibition (HI), complement fixation (CF), and neutralization tests. Trypsinized, centrifuged packed cells from whole C3Hf mouse embryos were diluted 1:200 in M 150 medium containing 2% calf serum, with penicillin and streptomycin, and seeded into culture tubes or 32-oz prescription bottles. The medium was changed after 2–3 days. When confluent cell sheets had developed, the initiating medium was replaced by a maintenance fluid consisting of the same medium with 1% calf serum. The cultures were inoculated by adding 0.5 ml or 5 ml to the tubes and bottles, respectively, of undiluted pooled supernatants from previous passages of polyoma virus cultures. The medium was changed once or twice weekly. When cytopathic effects were seen or positive hemagglutination tests were obtained, the fluids were collected and stored at —60°C.

VIRUS INOCULATIONS. Virus-containing fluids were inoculated into the animals after assay by cytopathic effects in mouse embryo culture; the titer was 10^4 TCID_{50}/ml. Hamsters received 0.5 ml i.p. of pooled supernatants from a 5th and a 7th passage when they were 3 weeks old, i.e., 2 weeks after BCG. As polyoma virus can induce tumors in older hamsters, BCG was injected before polyoma, in the hope of obtaining a better preventive effect.

Mice received 0.06 ml of fluid from the 7th passage, by the same route, within 24 hr after birth. As polyoma virus does not
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induce tumors readily in older mice, it was given to newborn animals and BCG was administered later.

BCG. Lyophilized BCG was used. The bacilli were resuspended in distilled water and injected i.p., at a dose of 0.4 mg wet weight in 0.1 ml, into hamsters when 1 week old and into mice when 4 days old.

STREPTOMYCIN. As BCG induces progressive tuberculous disease in hamsters (8, 10), streptomycin was administered to some groups with a view to delaying or suppressing the tuberculous condition. The treatment was instituted 2 months after BCG inoculation, when signs of infection began to appear and BCG was presumed to have exerted its antitumoral effect. This treatment lasted 6 months. Each animal received an s.c. injection of streptomycin sulfate 3 times a week, the dosage being gradually increased from 4 to 8 mg/injection. A 2nd course, of ten 8-mg injections over 2 weeks, was given 2 months later. A 3rd course, of 8 mg 5 times a week during 10 weeks, was started 1 month later.

SEROLOGY. Blood samples were obtained by inner canthus puncture from both hamsters and mice 3 weeks after polyoma inoculation. A 2nd sample of blood was taken from the surviving animals 2 months later in hamsters and 6 months later in mice. Each sample was centrifuged, and the serum was stored at −20°C. Hemagglutination, HI, and CF tests were performed essentially in accordance with the procedures described by Rowe and associates (14—16). Guinea pig RBC were used for hemagglutination. Neutralizing antibodies were evaluated by neutralization of cytopathic effects in vitro with mouse embryo cultures, after the method elaborated by Kalter and Hillis (9), with the use of Karber’s formula to calculate TCID<sub>50</sub> and antibody titers.

PATHOLOGY. All animals were autopsied. In many cases, the presence or nature of the polyoma lesions and tumors was established or confirmed by microscopic examination of tissues.

Results

HAMSTERS. One hundred and twenty-eight hamsters were divided into 6 groups and treated as shown in Table 1. Eight sucklings died in the 1st 3 weeks of life and were excluded from the experiment. Animals inoculated with polyoma virus alone developed characteristic changes and died at a comparatively early age. The lesions encountered were: (a) hemorrhagic blebs in the liver and lung, variously described in the literature as hemorrhagic cysts, vesicles or lesions, cystic angiomas, hemangiomata, hemangiosarcomas, and hemangiendotheliomas; many animals died as a result of these blebs rupturing into the peritoneal and, sometimes, the pleural cavity; (b) tumors, mostly subcutaneous sarcomas in diverse sites. Control animals in Group 2 seem to have benefited by streptomycin treatment, but the difference between this group and the 1st is not significant. Nevertheless, this point is being investigated (see Discussion).

With BCG, the incidence of malignancy was greatly reduced and survival was prolonged. This is especially obvious when the 50% survival time is considered, which is a better index than the average survival time. Hamsters without streptomycin (Group 3) died in a short period (Chart 1) of BCG infection, many of them without having had time to develop tumors; hence the figures do not give an exact picture of BCG protection against polyoma. What can be said for certain is that polyoma was retarded: the incidence was low (24%) at a time when most of the controls had already developed polyoma changes. The real effect is to be found in Group 4 (BCG + polyoma + streptomycin): here, the incidence was low, the difference of survival is highly significant, and the tuberculous
TABLE 2

<table>
<thead>
<tr>
<th>GROUPS*</th>
<th>NO. OF SERA</th>
<th>AVERAGE TITER OF ANTIBODIES (1ST SERUM) 1: . . . ± S.E.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 and 2. Polyoma</td>
<td>31</td>
<td>1289 ± 249</td>
<td></td>
</tr>
<tr>
<td>3 and 4. BCG + polyoma</td>
<td>49</td>
<td>2416 ± 353*</td>
<td></td>
</tr>
<tr>
<td>5 and 6. BCG</td>
<td>40</td>
<td>17 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

* The 1st serum was taken before streptomycin treatment; animals were still divided into only 3 groups.
* Difference significant: P < 0.02.
* Difference not significant.

TABLE 3

PARALLELISM OF THE 3 TYPES OF ANTIBODIES IN INDIVIDUAL HAMSTERS (GROUP 1)

<table>
<thead>
<tr>
<th>Antibody titers 1: . . .</th>
<th>Hemagglutination Inhibiting</th>
<th>Complement fixing</th>
<th>Neutralizing</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>80</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>640</td>
<td>60</td>
<td>723</td>
<td>2891</td>
</tr>
<tr>
<td>1280</td>
<td>80</td>
<td>1445</td>
<td>2891</td>
</tr>
<tr>
<td>1280</td>
<td>160</td>
<td>4096</td>
<td>4096</td>
</tr>
<tr>
<td>2500</td>
<td>160</td>
<td>5781</td>
<td>4096</td>
</tr>
<tr>
<td>2500</td>
<td>320</td>
<td>12390</td>
<td></td>
</tr>
<tr>
<td>5120</td>
<td>160</td>
<td>12390</td>
<td></td>
</tr>
</tbody>
</table>

Effect of BCG Infection on Polyoma

process was inhibited. Despite streptomycin, several hamsters died with tuberculous lesions, but much later than in Group 3. In the lower part of Chart 1, it can be seen that hamsters given only BCG died at a comparatively early period; here again streptomycin did markedly prolong life, although most animals ultimately died of tuberculous disorders.

All 3 types of antibodies were found to be increased in the groups with BCG + polyoma (Table 2). When these groups are compared with the groups given polyoma virus alone, the differences are statistically significant. However, Chart 2 shows that these differences are not very important, because the peaks in the distribution of antibody titers differ by only 1 dilution. Good parallelism was observed between the 3 types of antibodies: individuals with high or low titers for 1 type also had high or low titers for the other types. This is illustrated for Group 1 in Table 3; the same conclusion holds true for the other groups.

A comparison of antibodies in the 1st sample of blood with those in the 2nd sample is shown in Table 4. As CF and neutralizing antibodies always ran parallel to HI antibodies, they were measured in the 2nd serum of only certain animals, for a check. Not much information can be derived from Groups 1 and 2, because too few animals were still alive when the 2nd serum was taken (15/31), and the difference is not significant between the 2 sera. However, there was a tendency for antibodies to decrease, both in the individual animals and in the group. In Groups 3 and 4, the decrease was pronounced. It should be noted that the final point in these groups is about the same as that seen in Groups 1 and 2, suggesting that, with BCG, antibodies were produced in higher amounts than in controls without BCG, but fell to the same level after some time.

An interesting observation was made in hamsters not given BCG (Table 5): hamsters with less antibody response lived longer, and inversely, animals developing higher amounts of antibodies were as a rule the 1st to die. This can also be deduced from Table 4: in the 15 hamsters still alive 9 weeks after polyoma inoculation, the average titer of HI antibodies was 743, compared with 1801 for the 16 already dead at that time and with 1289...
for the group as a whole (Table 2). This is another indication that the animals developing more antibodies died earlier. In addition, the small number of hamsters that did not show polyomatosus lesions were all among those with very low or no antibodies, whereas the animals with higher titers of antibodies generally showed more severe changes.

In hamsters given polyoma + BCG (Groups 3 and 4), no correlation could be found in individual animals between the amount of antibodies and the development of polyoma and time of death.

MICE. Fifty-one mice were divided into 2 groups and treated as shown in Table 6. Two sucklings died the day after inoculation and were not considered in the results. Almost all the animals infected mice, but the differences are not statistically significant. When the 2nd serum was taken 6 months after the 1st, a number of the animals were dead, chiefly in the 1st group. Nevertheless, it seems that antibodies were markedly increased in this 2nd serum compared with the 1st, and this increase was greater in mice given BCG. No correlation could be found, in either group, between the amount of antibodies and the survival time of individual mice.

Discussion

It is apparent from the above results that BCG infection counteracted polyoma in hamsters, both by reducing the incidence of pathologic changes and by prolonging survival. This confirms our previous findings (11). BCG also stimulated the production of serum antibodies, a fact that had been established in other experimental systems (2, 6). Yet, this stimulation, although statistically significant, does not appear to be of great importance (Chart 2) and is not likely to be the cause of BCG protective effects. The fact that, among hamsters inoculated with polyoma virus but not given BCG, the animals with larger amounts of antibodies developed more extensive changes and died earlier is a further indication that the humoral antibodies considered are not responsible for resistance against tumors, a conclusion already inferable from the literature (5, 18, 19). The protection might be afforded by different antibodies, directed, not against the virus, but against antigens that were induced in cells rendered neoplastic by the virus. Evidence for such tumor antibodies in polyoma has been reported (5, 17, 19, 20). These antibodies would also be stimulated by BCG, hence the favorable action of the latter. Their presence and influence, as affected by BCG, are under investigation.

The finding that the hamsters with high titers of serum antibodies were less resistant may be explained by the reason that, in more susceptible animals, the virus was more active and evoked the production of both more antibodies and more tumors. As viral antibodies are not effective in preventing tumor growth, a little or more stimulation (as by BCG) is of no consequence. In contrast, for tumor antibodies, a small degree of stimulation may be critical and mean a significant difference in countering activity.

TABLE 4

**Comparison of HI Sup Antibodies in the 1st and the 2nd Serum of Hamsters**

<table>
<thead>
<tr>
<th>.Groups</th>
<th>No. of Hamsters</th>
<th>Average Titer of Antibodies 1st serum</th>
<th>2nd serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 and 2. Polyoma</td>
<td>15</td>
<td>743 ± 344</td>
<td>419 ± 177</td>
</tr>
<tr>
<td>3 and 4. BCG + polyoma</td>
<td>43</td>
<td>2381 ± 387</td>
<td>466 ± 100</td>
</tr>
<tr>
<td>5 and 6 BCG</td>
<td>40</td>
<td>17 ± 2.5</td>
<td>27 ± 3.5</td>
</tr>
</tbody>
</table>

* HI, hemagglutination inhibition
* In the animals that yielded a 2nd serum.
* Significance of difference between 1st and 2nd serum: P < 0.001.

TABLE 5

**Antibodies and Survival Time in Hamsters not Given BCG (Groups 1 and 2)**

<table>
<thead>
<tr>
<th>Animals in order of increasing amounts of antibodies</th>
<th>Average Tenors of Antibodies</th>
<th>Average Survival Time (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamsters No. 1-6</td>
<td>0 ± 0</td>
<td>327</td>
</tr>
<tr>
<td>Hamsters No. 7-12</td>
<td>113 ± 17</td>
<td>209</td>
</tr>
<tr>
<td>Hamsters No. 13-18</td>
<td>1173 ± 133</td>
<td>161</td>
</tr>
<tr>
<td>Hamsters No. 19-24</td>
<td>1433 ± 160</td>
<td>106</td>
</tr>
<tr>
<td>Hamsters No. 25-31</td>
<td>3291 ± 267</td>
<td>75</td>
</tr>
</tbody>
</table>

TABLE 6

**Effects of BCG on Polyoma in Mice**

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of Mice</th>
<th>Incidence of Polyoma</th>
<th>Survival Time Days</th>
<th>HI Antibody Titer 1st serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyoma</td>
<td>22</td>
<td>91%</td>
<td>174.3 ± 18.8</td>
<td>786 ± 59</td>
</tr>
<tr>
<td>Polyoma + BCG</td>
<td>27</td>
<td>89%</td>
<td>226.9 ± 19.2</td>
<td>960 ± 108</td>
</tr>
</tbody>
</table>

* HI, hemagglutination inhibition.
* No. of mice when 2nd serum was taken.
* Significance of difference: P = 0.055.
In mice, the effects were similar to those seen in hamsters, but were less pronounced. This lesser action is probably due to the BCG having been administered after the polyoma virus. It might be possible to obtain more protection by injecting the bacilli and virus at the same time or inoculating BCG at birth and polyoma a few days later, on the basis that virus strains of high activity can induce tumors in mice as old as 14 days (3, 18).

It remains to be seen whether the protective effect is caused by the infectious process of BCG or by an enhancement of defense responses that could be obtained with killed mycobacteria, as in Freund’s adjuvant, or with other agents. Fractions of BCG have been reported to show antitumoral activity (22), but we saw no such action of killed BCG on transplanted leukemia (11). The protective effect may also be referable, partly or wholly, to other mechanisms: stimulation of phagocytic activity (2, 12), interference between bacterial and viral infection, enhancement of hormone secretion, and others.

Streptomycin showed a tendency to counteract polyoma by itself (Table 1, Group 2). Although the differences in survival time were not significant, this point is under further investigation. Should this tendency prove to be a real effect, then streptomycin could act either against the virus or against some other factor, such as superimposed infection. Thus the protection in Group 4 would be due to both BCG and streptomycin, the antibiotic not only impeding BCG infection but adding a further action against polyoma or other detrimental agent. However, streptomycin was studied so far, under various conditions, in tissue cultures infected with polyoma, and no inhibiting or other effect was found.

The tuberculous disease resulting from BCG in hamsters was hindered by streptomycin, but was not completely suppressed. Several animals ultimately died of this infection. Either the treatment was too moderate or there developed some lines of bacilli that were resistant to the antibiotic. The latter possibility is not improbable, because in animals that died of polyoma during a certain period of the streptomycin treatment, the tuberculous process had regressed (Group 4); but it later reappeared in some animals and progressed eventually until death. It might be possible to devise some form of treatment that would eliminate tuberculous disease while retaining the beneficial influence of BCG.

References

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