BACTERICIDAL ACTIVITY OF SERUM FOR KLEBSIELLA RHINOSCLEROMATIS: STUDIES ON SERUM FROM A PATIENT WITH RHINOSCLEROMA AND SERA DEFICIENT IN ANTIBODY OR COMPLEMENT

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SUMMARY. The in-vitro bactericidal effect of serum for Klebsiella rhinoscleromatis was tested. Experiments with C2-deficient and hypogammaglobulinaemic human sera suggested that killing depended on activation of the classical complement pathway, although the alternative pathway probably amplified the effect. Serum from a patient with active rhinoscleroma, and another cured of the disease, showed normal killing.

INTRODUCTION

Rhinoscleroma is a granulomatous condition of the nasopharynx caused by chronic infection with Klebsiella rhinoscleromatis. The disease carries a high morbidity and is endemic in some parts of the world such as San Salvador and Egypt. There are no obvious climatic or genetic factors that predispose to the disease although some patients with it have particularly poor personal hygiene (Muzyka and Gubina, 1972).

Krasilnikov, Izraitel and Shimanovich (1974) have shown that the serum from some patients with active rhinoscleroma had poor in-vitro bactericidal activity against K. rhinoscleromatis. We have therefore investigated several factors in normal serum that might be involved in the killing of the organism and have also tested whether there were significant abnormalities in the serum of a patient with rhinoscleroma.

MATERIALS AND METHODS

Subjects

Serum was obtained from: patient no. 1 with active rhinoscleroma; patient no. 2, cured of rhinoscleroma a year previously; three patients with adult-onset hypogammaglobulinaemia—two of these had unrecordable levels of serum immunoglobulin (IgG <50, IgA and IgM <5 mg/100 ml) and the other was treated with injections of gammaglobulin and had a serum IgG of 270 mg/100 ml but unrecordable IgA and IgM; a patient with homozygous C2 deficiency. Serum was also obtained from healthy laboratory staff. In all cases, the serum was separated within 30 min of venesection and then stored at −70°C.

Bactericidal tests

Isotope-release method. K. rhinoscleromatis was isolated from the nose of patient no. 1 with rhinoscleroma and maintained on Columbia Blood Agar (Oxoid) slopes at 4°C. The bacteria were incubated for 18 h at 37°C in Nutrient Broth (Oxoid) containing uniformly labelled 14C D-glucose of specific activity 300 mCi/mmol (Radiochemical Centre, Amersham, Bucks) 1 μCi/ml. The bacteria were harvested by centrifugation at c. 1000 g for 10 min and washed

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three times in phosphate-buffered saline (PBS). The concentration of the suspension was adjusted to give an optical density of 0.06 at 540 nm when read against distilled water. This suspension contained approximately 10,000 cpm/100 μl.

Serum was diluted in PBS to the required concentration to give a final test volume of 1 ml. Tubes were pre-incubated at 37°C in a water bath for 5 min. The test was initiated by the addition of 100 μl of the 14C-labelled bacterial suspension and terminated by placing the tubes in ice. The samples were centrifuged at c. 2000 g at 4°C for 15 min; 500 μl of the supernate were removed and the radioactivity was measured in 5 ml of Triton X-100-toluene scintillation fluid, 1:2 (v/v). A control sample of the bacterial suspension in PBS was included in each experiment to give a background release of isotope with time. The value from the control sample was subtracted from the experimental cpm obtained in the presence of serum. Results were expressed as a percentage of the total cpm added.

**Plating technique.** *K. rhinoscleromatis* was seeded into 10 ml of Nutrient Broth (Oxoid) and cultured at 37°C for 18 h. The bacteria were harvested by centrifugation at c. 1000 g for 10 min, removing the supernate and resuspending in 5 ml of tissue-culture medium. The bacteria were counted, and the count was adjusted to 5 × 10⁷ organisms/ml immediately before use. Duplicate tubes were set up. To each tube was added 0.1 ml of bacterial suspension, 0.1 ml of serum and 0.8 ml of culture medium. The cultures were then incubated for 1 h at 37°C. A 25-μl portion of the culture was then removed and added to ice-cold PBS to make 1 in 200 and 1 in 2000 dilutions. Duplicate 25-μl portions of these two dilutions were spread on nutrient agar and the colonies were counted after overnight incubation at room temperature.

**RESULTS**

Fig. 1 shows the rate of release of 14C from *K. rhinoscleromatis* by 10% serum from four normal subjects; 70% of the isotope was released by 60 min. Fig. 1 also shows that the serum

![Graph showing release of isotope with 10% serum.](image-url)
from patient no. 1 with active rhinoscleroma behaved normally. In contrast, sera from the patients with primary hypogammaglobulinaemia released isotope much more slowly.

The fig. 2 shows the $^{14}$C release by increasing concentrations of serum from normal subjects and patients after incubation for 15 min. In 13 normal subjects, 50% (mean of range 30–60%) release of $^{14}$C was achieved with 30% serum. The serum of a patient cured of rhinoscleroma 1 year earlier showed greater release than the normal range for concentrations of serum over 20%. The serum from patient no. 1 with active rhinoscleroma showed normal release up to 30% serum, although with higher concentrations the release was a little below the normal range.

Fig. 3 shows the effect on normal and hypogammaglobulinaemic serum of inhibiting factor B of the alternative complement pathway by heating at 50°C for 20 min (Martin and Lachmann, 1977). However, treating serum in this way also inactivated most of the C2 present and probably much of the C1. Patients with primary hypogammaglobulinaemia have low serum C1 levels so there is likely to be very little C1 activity left after heating. It is therefore difficult to interpret the experiments with hypogammaglobulinaemic serum although the shape of the response curve (fig. 3) suggests that the alternative pathway amplifies the release of isotope.

Fig. 4 shows the results of a bactericidal assay by the plating technique in the presence of serum from a normal subject and from patient no. 1. There was almost complete killing of the organisms by 10% (v/v) patient serum at 30 min. Heating normal serum at 56°C for 30 min abolished bactericidal activity. Normal bactericidal activity was also shown by the serum from patient no. 2 who had previously been cured of rhinoscleroma. Serum from normal subjects killed virtually all the organisms in the assay after a 30-min incubation when diluted down to 5%.

**DISCUSSION**

We have shown that serum from normal subjects and patients with active or inactive rhinoscleroma are bactericidal for *K. rhinoscleromatis*. The killing is complement dependent.
Fig. 3.—Isotope release with increasing concentrations of C2-deficient, hypogammaglobulinaemic and normal sera. Serum from: e = patient with untreated hypogammaglobulinaemia; f = same as (e) but heated at 50°C for 20 min; g = normal subject; h = same as (g) but heated at 50°C for 20 min; i = C2-deficient patient.

Fig. 4.—Bactericidal action (plating technique) with 10% normal serum (■), 10% normal serum inactivated at 56°C for 30 min (○), 10% serum from patient no. 1 with active rhinoscleroma (□), and PBS control without serum (△).
and our findings with hypogammaglobulinaemic and C2-deficient sera suggest that this is mediated mainly by the classical complement pathway. The wide range of 14C release seen with sera from different normal subjects, particularly at low concentrations, is probably due to the variable serum concentrations of antibody to \textit{K. rhinoscleromatis} or \textit{Klebsiella} spp. (Michael, Whitby and Landy, 1962). Most normal subjects have a low titre of antibody to \textit{K. rhinoscleromatis} when this is tested by a fluorescent technique for specific IgG on the surface of the organism (unpublished observations). In this context, it is interesting that serum from the hypogammaglobulinaemic patient on treatment with human IgG immunoglobulin showed a relatively greater release of isotope than that of the untreated virtually agammaglobulinaemic patient, when tested at low concentration (fig. 1). This suggests that specific antibody and the classical pathway are crucial in initiating cell-wall damage. Patients with severe hypogammaglobulinaemia probably have a little circulating IgG antibody to \textit{Klebsiella} spp. although this may be difficult to demonstrate. This could partly explain why significant release of isotope was seen only at relatively high concentrations of serum from a severely hypogammaglobulinaemic patient (fig. 3). However, the pattern of this response, with a rapid rise in isotope release between serum concentrations of 30 and 40\% suggests amplification by the alternative complement pathway. Patients with hypogammaglobulinaemia usually have raised serum levels of factor B (Nakajima, Nihei and Hyodo, 1978) which might account for this phenomenon.

Although direct comparison between our standard bactericidal assay by the plating technique and the 14C release assay cannot be made, the latter probably does represent bacterial cell-wall damage. The release of 14C is probably the result of the formation of complement-dependent holes in the membrane. These holes can be seen by electronmicroscopy when \textit{K. rhinoscleromatis} is exposed to normal fresh serum (R. Dourmashkin, unpublished).

Some bacteria are able to activate the alternative complement pathway directly without involving the classical pathway (Schreiber et al., 1979). Schenkein and Ruddy (1981) have also shown that IgG antibody is involved in activation of the alternative pathway by zymosan. However, neither of these mechanisms seems to operate in the release of isotope from \textit{K. rhinoscleromatis} because of the negative results with C2-deficient serum.

The killing of \textit{K. rhinoscleromatis} is similar to that described for \textit{Shigella} by Reed and Albright (1974). They found that the killing of various strains of \textit{Shigella} depended on activation of the classical pathway by specific antibody, with amplification by the alternative pathway.

Serum from the patient with active rhinoscleroma gave a normal response in the 14C release assay when the concentration of serum was below 40\%. The subnormal response with higher concentrations of serum may be similar to that observed by Krasilnikov et al. (1974) because they were using serum concentrations of about 50\% in their bactericidal assay. The reason for the inhibition at high serum concentrations is not known but may be due to a blocking effect by high concentrations of specific antibody. However, this is unlikely to be of importance \textit{in vivo} because the concentration of serum proteins at mucosal surfaces can be very low.

The question still remains why patients with rhinoscleroma develop granuloma and how \textit{K. rhinoscleromatis} escapes from the bactericidal effects of antibody and complement before being phagocytosed by cells in the nasal mucosa. Our findings indicate that, to answer this question, attention should be directed to the micro-environment of the nasopharynx.

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