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THE EFFECT OF BACTERIAL CONTAMINATION OF IRRITANTS ON RESULTS IN RAT PAW OEDEMA EXPERIMENTS

BY

D. GREEN

From the Department of Pharmacology, John Wyeth & Brother Ltd., Institute of Medical Research, Taplow, Maidenhead, Berks.

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The inflammatory response induced in the hind paws of rats by the injection of irritant substances has been used for many years as a laboratory model for the evaluation of anti-inflammatory drugs. Many different irritant substances have been used for this purpose, including dextran, albumen, brewer's yeast extract, formaldehyde solutions and mustard. In many cases, however, it is not possible to demonstrate antagonism of the oedema induced by these substances with therapeutic dose levels of drugs known to show anti-inflammatory activity in man. The case against using a laboratory model of inflammation which requires the use of near-toxic doses of anti-inflammatory drugs has been clearly explained by Wagner-Jauregg, Jahn & Büch (1962).

In order to overcome these disadvantages, several groups of workers investigated the possibility of using various bland adsorbent substances as irritants. Hillebrecht (1954) described a method using kaolin to provoke oedema and Wagner-Jauregg & Jahn (1963) described a similar test using Aerosil (colloidal silicon dioxide). In both cases the development of oedema could be antagonized by " therapeutic " doses (doses which do not produce acute toxic manifestations) of most recognised anti-inflammatory drugs. The response to a given dose of kaolin varies widely from day to day, however, and variations in barometric pressure and humidity have been shown by Haberland (1961) to be at least partially responsible for this variability.

Winter, Risley & Nuss (1962) used the λ-fraction of carrageenin, a mucopolysaccharide extracted from the red alga *Chondrus crispus*, to provoke oedema of the rat hind paw. They found that this oedema was readily antagonized by steroid and nonsteroid anti-inflammatory drugs.

It seemed probable that suspensions of kaolin or carrageenin might easily become contaminated unless aseptic precautions were taken during preparation, storage and administration. All bacteria provoke an inflammatory response when they are administered parenterally and random contamination might lead to widespread variability in the rats' responses. We wished to attempt to assess the value of aseptic precautions in combating this source of variability.

METHODS

A sample of powdered carrageenin was sterilized by exposure to ethylene oxide for 48 hr. Solutions of 0.25, 0.5, 1 and 2% w/v were prepared in injection vials, using sterile 0.9% saline as diluent. Two vials were prepared at each concentration and one of each pair was immediately opened and allowed to stand in the laboratory for 4 days before use. At the end of this time, samples were taken from each of the closed and opened vials of 2% carrageenin for total bacterial counts to be made.

Twenty-four female rats (Wistar derivatives) in the weight range 160–180 g were randomly divided into groups of six rats each. The hind foot volumes of all the rats were determined accurately, using a volume recording device similar in principle to that described by Lenče (1962).

Carrageenin solutions (0.1 ml. volumes) were injected subcutaneously into each hind paw so that each rat received a dose of one of the four strengths of sterile carrageenin in the right hind foot and 0.1 ml. of the same concentration of carrageenin from the opened vials in the left hind foot. Aseptic precautions were observed when injecting the carrageenin. The vial tops were swabbed with 70% ethanol and two sterilized syringes and needles were used for each group. The plantar surfaces of the rats' hind feet were thoroughly cleaned with 70% ethanol before each injection. The hind foot volumes were redetermined 5 hr later. The mean change in foot volume and the standard error of the mean were calculated for each treatment.

A similar experiment was carried out using sterile and contaminated kaolin. Vials containing 5, 10, 20 and 40% w/w kaolin in 0.9% saline were sterilized by autoclaving. One vial of each concentration was opened and allowed to stand in the laboratory for 4 days. Samples were then removed for culture from the opened and unopened vials containing 40% kaolin. Groups of six rats received sterile kaolin in the right hind feet and contaminated kaolin in the left hind feet. The design of this experiment was in other respects similar to that described above for carrageenin.

In a third experiment, we attempted to ascertain whether the presence of killed bacteria in the sample would provoke the same inflammatory response as was seen in the first two experiments when samples became contaminated with living bacteria. The hind foot volumes of twelve rats were determined as before The animals were then dosed as follows:

Group 1: right hind feet, 0.1 ml. sterile kaolin, 25% w/w; left hind feet, 0.1 ml. kaolin suspension, 25% w/w, which had been prepared sterile and then contaminated by adding 5×10^6 spores of *Bacillus subtilis* per ml.

Group 2: right hind feet, 0.1 ml. sterile kaolin, 25% w/w, to which had been added 10 mg/ml. of streptomycin sulphate and 10 mg/ml. of procaine penicillin; left hind feet, 0.1 ml. sterile kaolin, contaminated with 5×10^6 spores of *B. subtilis*; after contamination, 10 mg/ml. of streptomycin sulphate and 10 mg/ml. of procaine penicillin were added, in order to kill the bacteria; this sample was cultured in order to insure that there were no living organisms present.

RESULTS

Effect of contamination on carrageenin-induced oedema. The mean foot volumes of the four groups of rats which were dosed with sterile carrageenin in the right hind feet and contaminated carrageenin in the left hind feet are shown in the lower half of Table 1. The change in volume of the feet increased with the dose of carrageenin whether the carrageenin was sterile or not. The mean volumes recorded for the feet treated with unsterile carrageenin were 1.21- to 1.46-times higher than those recorded for feet treated with equal doses of sterile carrageenin. The probability of significance of difference between the means for groups dosed with the same concentrations of carrageenin was greater than 95% ($P<0.05$, as determined by Student's t-test) in every case except the groups dosed with 1% carrageenin ($0.05<P<0.1$).

Effect of contamination on kaolin-induced oedema. The mean foot volumes of the rats dosed with sterile kaolin in the right hind feet and contaminated kaolin in the left hind feet are shown in the upper half of Table 1. These results were similar to those seen with

TABLE 1

THE EFFECT OF RANDOM BACTERIAL CONTAMINATION ON OEDEMA INDUCED BY CARRAGEENIN AND KAOLIN

Each value is the mean increase in paw volume of six rats and the standard error of the mean, recorded 5 hr after the injection of irritants

Irritant	Concentration (%)	Paw volume increase (ml.)	Total bacterial count (per ml.)
Sterile kaolin	5	0.440 ± 0.0327	–
	10	0.583 ± 0.0424	–
	20	0.593 ± 0.0546	–
	40	0.753 ± 0.0424	Nil
Unsterile kaolin	5	0.690 ± 0.0297	–
	10	0.948 ± 0.0777	–
	20	0.970 ± 0.0581	–
	40	1.155 ± 0.0452	10^4
Sterile carrageenin	0.25	0.600 ± 0.0321	–
	0.5	0.760 ± 0.0462	–
	1.0	0.833 ± 0.0458	–
	2.0	0.925 ± 0.0539	Nil
Unsterile carrageenin	0.25	0.845 ± 0.0474	–
	0.5	0.927 ± 0.0329	–
	1.0	1.012 ± 0.0696	–
	2.0	1.348 ± 0.0619	$>10^5$

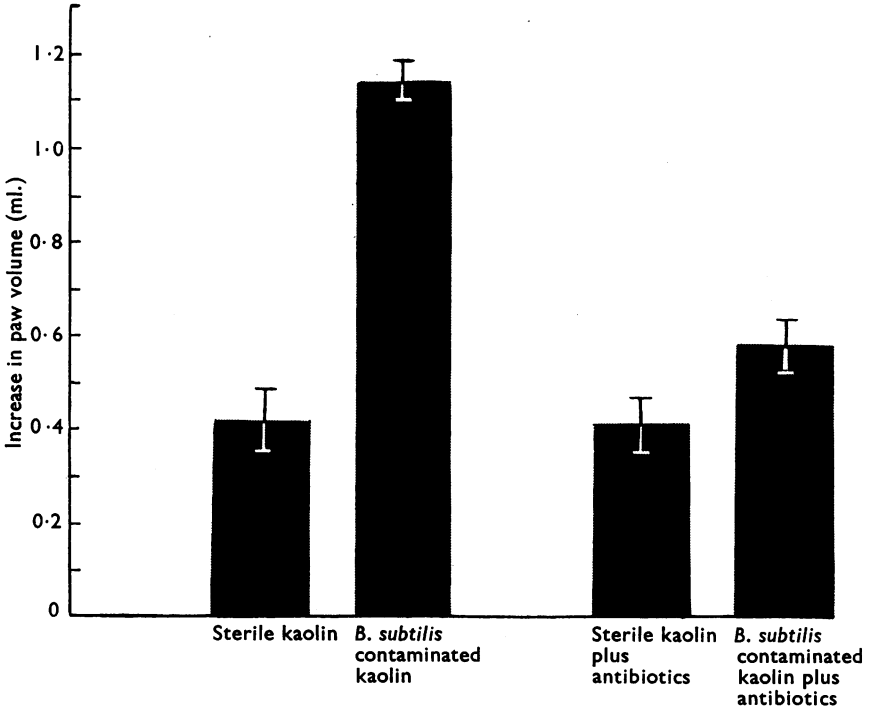

Fig. 1. The effect of living and killed bacteria (*B. subtilis*) on the volume of oedema fluid developed 5 hr after an injection of 0.1 ml. of treated 25% w/w kaolin suspension. Vertical lines show the standard errors of the means. The *B. subtilis* contaminated kaolin had more than 10^6 organisms/ml. The contaminated kaolin with antibiotics was sterile when cultured.

carrageenin, but the effect of bacterial contamination was more marked. The mean foot volumes recorded for the feet dosed with contaminated kaolin were 1.53- to 1.64-times greater than those recorded for feet dosed with a similar amount of sterile kaolin. The probability of significance of difference between the means for groups dosed with the same concentrations of kaolin was greater than 99.9% ($P<0.001$) in every case.

Effect of killed bacteria on kaolin-induced oedema. The effect of deliberate contamination of kaolin with spores of *B. subtilis* was clear. The mean increase in volume of the hind feet dosed with contaminated kaolin was 2.78-times greater than that of the opposite hind feet, which were dosed with sterile kaolin. These results are shown as a histogram in Fig. 1.

The mean increase in volume of the feet treated with sterilized kaolin with added streptomycin sulphate and procaine penicillin did not differ significantly from that recorded for feet treated with sterile kaolin only. Deliberate contamination of a sterile kaolin suspension containing antibiotics with spores of *B. subtilis* before injection into the opposite hind feet gave a much smaller increase in mean foot volume than did contamination in the absence of antibiotics. Nevertheless, the killed *B. subtilis* provoked a response which was 1.39-times that which occurred in the group dosed with sterile kaolin plus antibiotics ($0.02<P<0.05$).

No viable organisms could be demonstrated by culturing the sample of contaminated kaolin which contained antibiotics.

DISCUSSION

In attempts to discover a reliable test for screening substances with potential anti-inflammatory activity we used experimental models which involved the induction of oedema with either kaolin or carrageenin. Both these models proved, in our laboratory, to give rise to rather variable responses. Groups of animals injected late in the day reacted to the irritant substances more strongly than groups which were injected earlier. As atmospheric conditions were kept almost constant within the animal houses and laboratories (temperature, $20\pm2°$ C; relative humidity, 60 ± 5%) it seemed unlikely that this variability was brought about by changing weather circumstances, as described by Haberland (1961).

The property of micro-organisms to cause an inflammatory reaction when injected is widely known, and several animal models of arthritis which involve the parenteral administration of micro-organisms are mentioned in Gardner's (1960) review. The possibility that suspensions of kaolin and carrageenin might readily become contaminated in the laboratory and that this might in turn lead to a large change in irritant potentialities lead us to evaluate the use of aseptic technique, in the first two experiments described above. As the results indicate a very marked influence of random bacterial contamination of kaolin and carrageenin on the volumes of oedema fluids produced, we feel that aseptic technique is worth while if these substances are used as models for the evaluation of anti-inflammatory activity.

The third experiment was designed to discover whether living bacteria were necessary to produce the enhancement of irritant activity of kaolin, or whether killed bacterial debris and toxins would be sufficient. Although the kaolin suspension containing killed *B. subtilis* plus antibiotics was significantly more active in producing oedema ($0.02<P<0.05$) than the sterile kaolin with added antibacterials, it is clear that living bacteria produce a much greater potentiating effect.

SUMMARY

1. It has been shown that the volume of oedema induced in rats' hind paws by standard doses of kaolin or carrageenin is greatly increased if the sample becomes contaminated by living bacteria.

2. It is suggested that aseptic precautions should be observed when preparing, storing and injecting these substances during tests for anti-inflammatory activity.

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