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Studies on Bacterial Pyrogenicity

III. Specificity of the United States Pharmacopeia Rabbit Pyrogen Test[1]

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ABSTRACT

MARTIN, WILLIAM J. (University of Utah, Salt Lake City), AND STANLEY MARCUS. Studies on bacterial pyrogenicity. III. Specificity of the United States Pharmacopeia rabbit pyrogen test. Appl. Microbiol. **12**:483-486. 1964.—The specificity of the first or "presumptive" portion of the USP rabbit pyrogen test was investigated by use of a new absolute standard of reference. The reference standard was a 0.9% sodium chloride solution prepared to be pyrogen-free. Details of the preparation were described. The hypothesis was explored that the temperature response of rabbits after intravenous injection of the standard solution was independent of exogenous pyrogen. Reactions observed among the rabbits in our colony allowed a classification of these animals ranging from "consistently reliable" to "consistently unreliable." Details of the experimental results and implications for pyrogen testing are discussed. The recommendation was made that all rabbit test animals be "screened" in sham and actual tests before being used for pyrogen testing.

Contaminating bacteria constitute the only significant source of fever-inducing or pyrogenic agents in parenteral substances (Seibert, 1925; Probey and Pittman, 1945; Marcus, Anselmo, and Perkins, 1958; Marcus, Anselmo, and Luke, 1960). To test for potential pyrogenic capacity in any material designed for parenteral use, the rabbit pyrogen test described in the *Pharmacopeia of the United States* (1960) is a legal requirement.

The USP test is a two-part procedure. The first part may be called the presumptive test. In this procedure, three rabbits are injected intravenously (iv) with the substance under test. If no rabbit shows a temperature rise of greater than 0.6 C and the total rise in temperature for the three rabbits is no more than 1.4 C, the substance is adjudged nonpyrogenic.

Unfortunately, there is little common knowledge that this presumptive portion of the USP test is designed for maximal sensitivity at the expense of specificity. In other words, a positive presumptive test may be obtained despite the nonpyrogenic nature of a parenteral substance.

Because the rabbit test animal is notoriously unstable with regard to temperature regulation (Moore, 1918; Cotui and Schrift, 1942; McClosky et al., 1943; Probey and Pittman, 1945; Moliter et al., 1946; Grant, 1950), experiments were undertaken to determine, over an extended period, how reliable in pyrogen testing were the rabbits in our colony. In other words, a study was undertaken of the specificity of the presumptive test for pyrogens.

[1] This paper was taken in part from a dissertation submitted to the Graduate School, University of Utah, in partial fulfillment of requirements for the M.S. degree.

MATERIALS AND METHODS

Preparation of pyrogen-free saline. Figure 1 schematically illustrates the preparation of pyrogen-free (PF) (as contrasted to nonpyrogenic) reference solution. The tin-lined metal still shown was used in the preparation of the distilled water. After running the still for about 20 min, water was collected aseptically into a flask previously heated at 250 C for 2 hr. Granular NaCl in a weighing bottle, empty weighing bottles, hypodermic needles in glass tubes, aluminum foil-wrapped syringes, graduated cylinders, and Erlenmeyer flasks were heated in an oven at 250 C for 2 hr. The NaCl was weighed out aseptically into one of the tared, previously heated weighing bottles, and was added to measured amounts of the aseptically collected water. The flask containing the solution (usually 2 liters per preparation) was again covered with the

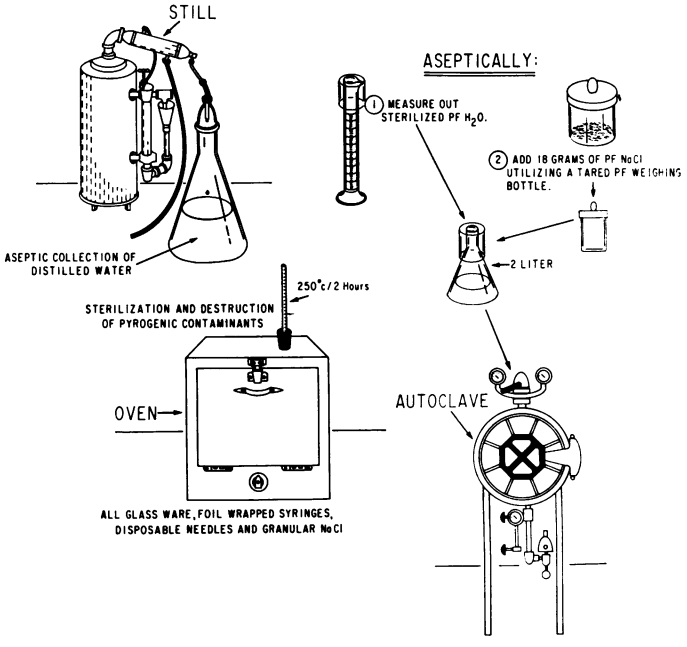

FIG. 1. *Preparation of pyrogen-free reference solution, 0.9% NaCl.*

previously oven-sterilized foil and beaker. The solution was then autoclaved at 120 C for 15 min.

The procedure shown in Fig. 1 operationally defines a method for preparing a pyrogen-free, sterile solution of 0.9% sodium chloride to be employed as a rabbit assay or pyrogen test control.

Animals used in testing. Previously tested male and female rabbits obtained from a local source were used for the experiments. Rabbits weighed between 3 and 5 kg, and were caged individually or in pairs in a constant-temperature room. Male and female rabbits were separated. Pyrogen tests were carried out according to techniques described in the *Pharmacopeia of the United States* (1960), with the following exceptions. All glassware was boiled in detergent solution, rinsed with nonpyrogenic distilled water, and sterilized by heating in an oven at 250 C for 2 hr; PF saline solutions to be tested were at room temperature rather than at 37 C; temperature recordings were taken prior to injection at 15 min, 1, 2, and 3 hr after injection. The results were interpreted as per the USP.

In each experiment, except where rabbits were sham-tested, all rabbits were injected exclusively with PF saline.

Temperature recording. Rectal temperatures were measured with an electric-type thermometer operating on the thermocouple principle (model TE3; Ellab Instruments, Copenhagen, Denmark). This instrument was checked periodically against water-bath controls.

RESULTS

Results after iv injection of PF saline in rabbits suitable and unsuitable for pyrogen test use. Table 1 shows results from an experiment with PF saline injected into nine rabbits deliberately chosen from a larger group of animals which had been previously tested at intervals, and which was found to contain animals varying in reliability for use in the pyrogen test. The nine rabbits were chosen for this experiment on the basis of their records of relative suitability or unsuitability for test use. By comparing the data in the maximal temperature change column in Table 1, it is seen that the rabbits yielded results which mark the animals as either suitable or unsuitable for pyrogen test use, because of the repetition of patterns previously displayed. For the sake of convenience, these nine rabbits were divided into three groups. The representative "suitable" rabbits comprise the numbers 44B, 5A, and 3C; "unsuitable" rabbits comprise the numbers 4A, 22C, and 33B; rabbits 22A, 2B, and 1B comprise a representative middle group, with maximal temperature changes somewhere between the suitable and unsuitable groups. The suitable rabbits represent the three animals that yield both an individual and a group maximal temperature change response which agrees with the USP definition. The middle group of unsuitable rabbits shows a group maximal temperature change of 1.6 C which is 0.2 C greater than the 1.4 C allowable total temperature response for the three-rabbit test, whereas the last group of unsuitable rabbits shows maximal temperature responses of greater than 0.6 C for each rabbit.

TABLE 1. *Results after intravenous injection of PF saline in rabbits suitable or unsuitable for pyrogen test use*

Determination	Rabbit no.	Control temp	Time after injection				Maximal temp change
			15 min	1 hr	2 hr	3 hr	
		C	C	C	C	C	
Consistently reliable	44B	37.8	37.8	37.9	37.8	37.7	+0.1
	5A	37.3	37.5	37.6	37.6	37.6	+0.3
	3C	38.0	38.0	38.0	38.1	37.9	+0.1
	22A	37.7	37.9	38.1	38.2	38.2	+0.5
	2B	37.1	37.2	37.4	37.5	37.6	+0.5
	1B	37.8	38.2	38.3	38.2	38.4	+0.6
Unreliable	4A	37.0	36.9	37.4	37.9	37.8	+0.9
	22C	38.0	38.4	38.4	38.3	38.8	+0.8
	33B	37.4	37.2	37.5	37.9	38.1	+0.7

Summary of results of repeated pyrogen tests with rabbits. The procedure of injecting PF saline into previously sham-tested rabbits was done repeatedly on a random group of test rabbits (Table 2). The criteria for the classification of the rabbits in Table 2 are based only on the individual responses of each rabbit. That is, if an individual rabbit showed a maximal temperature change from the preinjection temperature of 0.6 C or greater, then this animal was considered unsatisfactory. The number of unsatisfactory results obtained with each rabbit determined its group classification. The classification of these rabbits into the four groups shown was an arbitrary one, with the consistently reliable and totally unreliable groups being self-explanatory. The decision to place a rabbit in either a usually reliable or a usually unreliable group was also an arbitrary one. An animal was placed in the usually reliable group if it gave a satisfactory assay result in more than half of the total number of pyrogen tests in which it was used. Conversely, a rabbit was placed in the usually unreliable group if it gave false-positive reactions in at least half the tests in which it was employed. Of 31 rabbits, 13 gave consistently reliable results in three or more tests, and five of these

TABLE 2. *Summary of results of repeated pyrogen tests with rabbits**

No. of rabbits	Group designation
13 (11♀, 2♂)	Consistently reliable
5 (5♀)	Consistently unreliable
10 (6♀, 4♂)	Usually reliable
3 (3♀)	Usually unreliable

* Total of 31 rabbits (25 females, 6 males), randomly supplied from one source, were tested three to eight times.

animals, in four tests, gave totally unreliable results (Table 2). It is of interest that four of the five animals in this second group were unstable, even when sham-tested. These animals were discarded and, on the basis of the evidence collected, it was assumed that these discards represented members of a group of rabbits that could be termed "consistently unreliable." If it is assumed further that in such a (consistently unreliable) population the incidence of false positives in any five tests with the animals was 95% (19 of 20 assay results with members of this group were "unsatisfactory"), then the probability of detecting an animal representative of this group in any one test is 0.95; in two consecutive tests the probability is 0.9975; and in three consecutive tests, 0.999.

Of the 31 rabbits which were tested, 10 usually yielded negative results. That is, in five or more tests, each of these rabbits recorded one or two unsatisfactory assay results. On the other hand, 3 of the 31 rabbits which were tested five or more times were found to be usually unreliable, because three unsatisfactory results were obtained with each animal (Table 2).

Summary of results of repeated pyrogen tests with different group designation. On the basis of the foregoing results, as well as data collected with other rabbits, 28 animals in the test colony were assigned a group value, i.e., consistently reliable, usually reliable, and usually unreliable.

Table 3 gives cumulative results from 30 experiments in which PF saline was injected iv into these 28 rabbits divided into the three groups previously described. In these experiments, the three selected groups of rabbits were repeatedly injected with the PF saline reference solution to determine the temperature stability of the animals. If any rabbit showed a 0.6 C or greater rise in temperature, the increase was considered to be independent of exogenous pyrogen or environmental stimulus and to represent a false-positive reaction.

The procedure of injecting PF saline into these three groups of rabbits was done repeatedly at either 48 hr or at weekly intervals over a series of ten tests.

TABLE 3. *Summary of results of repeated pyrogen tests with rabbits with different group designations*

No. of rabbits	Group designation	Test period	Results
9 (8♀, 1♂)	Consistently reliable	Every 48 hr for 3 weeks	Two females each yielded three unsatisfactory tests.
10 (8♀, 2♂)	Usually reliable	Once each week for 10 weeks	One male yielded two and one male yielded one unsatisfactory tests
9 (4♀, 5♂)	Usually unreliable	Every 48 hr for 3 weeks	Three males yielded a total of 11 unsatisfactory tests

The results obtained were not in accord with the hypothesis tested. Thus, two of nine animals previously classified as consistently reliable each yielded three false-positive reactions, and only three of nine animals grouped as usually unreliable gave false-positive reactions (Table 3). Furthermore, of the nine consistently reliable rabbits given PF saline injections at 48-hr intervals, seven gave satisfactory (negative) results, whereas two of these rabbits each yielded three unsatisfactory assay results. Of ten usually reliable rabbits, two gave one and two unsatisfactory results upon repeated weekly intravenous injections of PF saline. Of nine usually unreliable rabbits given PF saline injections at 48-hr intervals, six gave satisfactory results, whereas three animals recorded a total of eleven unsatisfactory results. One rabbit (C7) gave only a satisfactory sham-test result, and test results with PF saline were all unsatisfactory.

Summary of results of repeated iv injections of sterile PF saline over a 2-year period. The procedure of injecting PF saline into previously sham-tested rabbits was done repeatedly as part of a testing routine. The data for the number of false-positive results recorded over a 2-year period were collected from all rabbit pyrogen tests conducted over this time period. Of 940 rabbits tested to date, 157 recorded unsatisfactory temperature responses; that is, a 0.6 C or greater rise in temperature was obtained after injection of pyrogen-free solutions. Thus, 16.7% of 940 rabbits receiving PF saline over a 2-year period were recorded as yielding false-positive reactions in the USP pyrogen test.

DISCUSSION

It is readily recognized, although not explicit in the available literature, that two major variables contribute to unreliability in the rabbit pyrogen test or assay procedure. First, each animal used may show unpredictable variations in response from one test to the next. Second, the sample under test may contain concentrations of pyrogenic material which are less than detectable by some rabbits.

The problem of separating these variables for independent study was the major part of the work reported here. The concept was proposed that a completely pyrogen-free physiological solution of sodium chloride (0.9%) could be prepared and thereafter used to test the temperature stability of each test rabbit after intravenous injection. The preparation of a pyrogen-free standard was based on the hypothesis that the only pyrogenic material that contaminates a laboratory preparation of physiological saline is bacterial in nature; therefore, a solution prepared with material totally free from bacteria or preformed pyrogens will transcend the nonpyrogenic status; it will be pyrogen-free.

From the experimental results described it was shown

that (i) a PF saline reference standard may be prepared, and (ii), with this standard solution, members of a random group of rabbits may be shown to be satisfactory or unsatisfactory for pyrogen testing. Indeed, it was found over a 2-year period that, of a total of 940 rabbits injected with this PF saline reference standard, 783 rabbits always recorded satisfactory temperature responses, compared with 157 rabbits which recorded one or more unsatisfactory responses. These data indicate that, under local conditions, approximately 17% false-positive results may be expected when a random group of rabbits is used for pyrogen testing. This result compares favorably with that of McClosky et al. (1943), who found 8% false-positive reactions in 1,017 measurements.

It is apparent that the stress of repeated 48-hr injections of sterile PF saline into previously sham-tested rabbits is a satisfactory procedure for screening these animals for use in the USP pyrogen test. Under these conditions, two of the rabbits, which were previously considered consistently reliable (Table 3), showed temperature variations outside the prescribed limits for negative response.

Previously sham-tested rabbits injected with PF saline at weekly intervals also gave some variation in response, but less than with the animals given repeated 48-hr injection. By comparing results given in Table 3 on consistently reliable and usually reliable rabbits, one can see the greater effect of the stress of testing every 48 hr, as compared with the stress of weekly injections. This could indicate that some of the rabbits used for the weekly tests were potentially capable of giving unsatisfactory assay results if, in addition, they had been tested every 48 hr. On the other hand, if weekly injections had been carried out on the previously selected consistently reliable rabbits, the potential for one or more of these animals to yield an unsatisfactory result might have been concealed. However, the stress of the 48-hr injection of PF saline on these animals revealed two rabbits capable of yielding false-positive results.

It appears from these data that, to reliably test for the presence of pyrogens by the rabbit method, one must first screen the test animals. A recommendation suggested is that at least two sham tests plus three actual tests involving injection of a sterile PF reference standard at 48-hr intervals be used. Any animal showing an unsatisfactory result during this series should be discarded.

The basic and applied significance of these experiments seems apparent. First, to increase the specificity of the three-rabbit pyrogen test, it is necessary to insure that satisfactory test animals are employed. Obviously, if unstable rabbits are employed, the validity of the experimental effort is open to question. Second, in experiments involving endotoxin measurements, screening of the test rabbits should be employed to make results more valid.

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