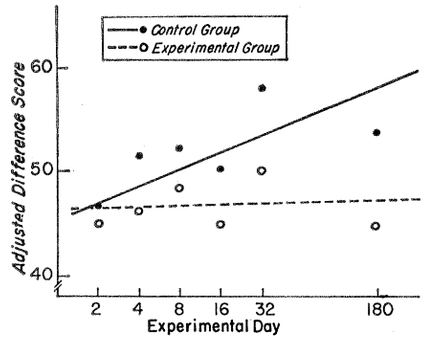

Fig. 2. Regression lines of best fit for control and experimental animals. The larger the adjusted difference score, the greater the effect of MS.

after administration. The difference between the "predicted" score and the score after drug administration was used for analysis. However, in order to avoid the use of negative scores in Fig. 2 and in Table 1, the mean predicted score was added to the mean of the adjusted difference scores. With the mean predicted score as a constant, we brought the adjusted score back to the magnitude of the square root of the area under the time-effect curve.

Figure 2 shows the mean adjusted difference score at each of the time intervals. The slopes of the experimental and control regression lines are 0.55 and 5.22, respectively. These slopes differ by means of t-test at an acceptable level of confidence ($t = 2.05$, $P < .05$). As compared to the control animals, the experimental animals had an attenuated effect to the drug, and the attenuation of the morphine effect is greater, the longer the time between the initial dose and the test dose. The control animals tested late in the experiment had a more intense reaction to the MS (5 mg/kg) than those control animals tested early (Fig. 2). This greater response over a period of time may have been due to the increase in weight of the animals. On day 1, the mean weight was 200 g, and on day 32 it was 350 g. (The weight was the same for control and experimental animals throughout the experiment.) Since the dose was in milligrams per kilogram of body weight, the heavier animals received more drug. This suggests that giving MS in doses of miligrams per kilogram does not equate animals of different weights.

Table 1. Mean adjusted difference scores for both control and experimental groups in experiment 2. On day 8, $P < .025$ (single-tail t-test) between control and experimental groups; and $P < .05$ (single-tail t-test) between the two experimental groups.

Group	Scores	
	Day 2	Day 8
Control	33.65	35.83
Experimental	35.30	29.20

In order to remove the confounding of the change in weights of the animals, we repeated the experiment for days 2 and 8. In this experiment, the weights of the animals were kept constant; that is, the animals in all groups tested were approximately the same age and weights, a condition achieved by giving the initial dose of 10 mg/kg at different times before the test day (Table 1). There was no evidence of tolerance on day 2 of the experiment, but there was marked evidence on day 8.

These results confirm the phenomenon of single-dose tolerance in the rat and indicate that, at these doses, the tolerance is not present 24 hours after the initial dose was given. In fact, the tolerance becomes more pronounced the longer the time interval between the two doses of the drug. The mechanism for this type of tolerance may be quite different from that after repeated large doses of MS.

One hypothesis that has been proffered is that this single-dose tolerance may be the result of an immune mechanism. Passive transfer experiments have suggested that this may be so (*4*). However, these experiments have not given consistent results. In fact, there is some evidence suggesting that there may be a potentiating factor in the serum of the animals previously made tolerant to MS (*5*). Thus the enigma of tolerance to this drug is not solved but only made more complicated by the results of this experiment.

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Nonspecific Staining: Its Control in Immunofluorescence Examination of Soil

Abstract. *Gelatin preparations were used to treat soil slides prior to addition of fluorescent antibody. Nonspecific staining was avoided, with no detectable interference to specific staining. Gelatin-rhodamine conjugates served to counterstain as well as to prevent nonspecific staining.*

Many applications of the fluorescent antibody (FA) technique are complicated by nonspecific staining reactions resulting from retention of fluorescent components of a conjugate by mechanisms other than known immunologic reactions. Nonspecific staining may interfere with the FA detection of pathogenic bacteria in host tissues in diagnostic procedures, particularly when the conjugates are undiluted or diluted only slightly (*2*). We have used the FA technique to study soil bacteria *in situ* and encountered problems due to nonspecific staining (*3*). We now report that we can control nonspecific staining in the immunofluorescence examination of soil preparations, and have applied this technique to some problems in diagnostic microbiology.

Gelatin solutions modified by partial alkaline hydrolysis prevented nonspecific staining. When the modified gelatin was conjugated to the fluorochrome dye rhodamine isothiocyanate (RhITC), the preparation provided additional desirable features as an effective counterstain. The gelatin apparently adsorbs to soil and tissue, blocking sites of nonspecific adsorption; the dye conjugated to the gelatin imparts an orange-brown background fluorescence to the soil or tissue in good contrast to the yellow-green of a fluorescein-labeled antibody.

A 2-percent aqueous solution of gelatin (*4*), adjusted to *p*H 10 to 11 with 1*N* NaOH, was autoclaved for 10 minutes at 121°C; the autoclaved solution was readjusted to the same *p*H. The gelatin was conjugated by (i) dis-

solving rhodamine isothiocyanate (*5*) in a minimum amount of acetone to provide 8 μg of dye per milligram of gelatin, (ii) adding this to the gelatin solution, and (iii) holding it overnight with gentle stirring. A 25-ml volume of gelatin solution was used. Samples of 10 to 15 ml of the mixture of gelatin and dye were washed through a gel (Sephadex G-25, coarse) column 2 by 30 cm at room temperature with phosphate buffer at *p*H 7.2. Conjugated gelatin-RhITC separated as a mobile red band which emerged in about 1.8 times the sample volume. Merthiolate was added (1 : 10,000), and the conjugate was distributed at once into 5-ml volumes and stored at −20°C.

The gelatin-RhITC conjugate was used principally for the examination of contact slides during study of *Rhizobium japonicum* in soil. Microscope slides were buried in the soil, and after recovery one side was prepared by rinsing, air drying, and heat fixing (*3*). The gelatin-RhITC conjugate (0.5 ml) was then added to flood approximately half of the surface, and the slide was placed at 60°C until the gelatin dried as a film. After the film cooled, the specific antibody-fluorescein isothiocyanate (FITC) conjugate was added to overlay the gelatin film, and the preparation was kept for 45 minutes in a moist chamber. Unreacted conjugates were washed off with *p*H 7.2 buffer, the slide was submerged in water for 15 minutes and blotted dry, and a cover slip was mounted on Difco FA mounting fluid. Equipment for immunofluorescence microscopy and photomicrography has been described (*3–5*).

Fields demonstrating the effect of gelatin-RhITC treatment are shown in Fig. 1. All slides represented in the photomicrographs were prepared after burial in sterile silt loam soil inoculated with *Rhizobium japonicum*. Without gelatin treatment (Fig. 1, a and b) most microscopic fields have extensive areas that fluoresce yellow-green. Nonspecific staining also resulted in undesirable dark to bright green background films in many fields. These problems were not encountered in all fields because specifically stained bacteria separated from soil particles may be readily distinguished against a dark background (Fig. 1a, center; Fig. 1b, lower right). The study of bacteria in soil of FA techniques in the presence of nonspecific adsorption is limited by poor contrast, masking effects, and the likelihood of artifacts.

Table 1. Results of immunofluorescence examination of soil contact slides after prior treatment with rhodamine isothiocyanate (RhITC)-gelatin conjugates of different ratios of due to protein. Slides were taken from sterile soil inoculated with *Rhizobium japonicum* 61A72 and stained with fluorescein-labeled (FITC) antibody to that bacterium.

RhITC in gelatin (μg/mg)		Microscopic features		
Added	Incorporated	Background*	Soil particles	Bacteria
0.0†		Black	Dark gray; barely visible	Bright yellow-green (FITC)
0.5	0.2	Black	Faint orange	Bright yellow-green
2.0	1.0	Dark	Dull orange	Bright yellow-green
4.0	2.7	Dark	Orange	Bright yellow-green
6.0	4.0	Dark	Orange-red	Bright yellow-green
10.0	7.2	Dark	Bright orange	Bright yellow-green
12.0	8.2	Faint orange	Bright orange	Bright yellow-green
16.0	11.4	Light orange	Bright orange	Yellow-green
30.0	23.6	Orange	Bright orange	Dull yellow-green
36.0	26.8	Bright orange	Barely distinguishable	Yellow (FITC-RhITC)
120.0	80.1	Intense orange	Indistinguishable	Pale yellow

* Areas of microscopic field devoid of soil particles. † Gelatin only.

Use of gelatin-RhITC virtually eliminated limitations imposed by nonspecific adsorption, apparently without interfering with the specific staining reaction. Soil particles were coated with the gelatin conjugate and apparently retained this rather than the specific conjugate. When examined by fluorescence microscopy, the soil particles appeared orange-brown to red-brown and provided good contrast to the intense yellow-green specific fluorescence of the bacteria. Within the limitations of black and white film, Fig. 1 (c and d) illustrates some of the effects of gelatin treatment in the presence of specifically stained bacteria. The irregular white areas representing nonspecific adsorption seen in Fig. 1 (a and b) are practically eliminated. Sites of nonspecific localization of FITC fluorescence were encountered only rarely on soil contact slides properly treated with the gelatin conjugate.

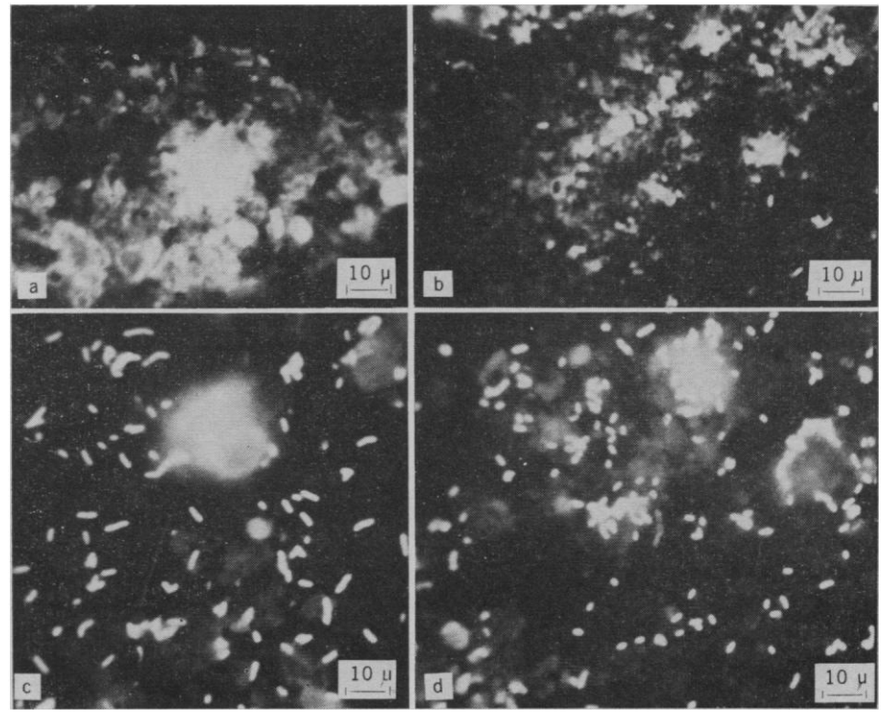

Fig. 1. Representative fields from slides that were in contact with soil and prepared for detection of *Rhizobium japonicum* by immunofluorescence. (a and b) Without prior treatment; soil particles that appear as diffuse white areas adsorbed the fluorescent antibody to *R. japonicum* and fluoresced dull green to yellow-green; bacteria can be distinguished in some areas away from soil particles. (c and d) Prior treatment with gelatin-rhodamine isothiocyanate conjugate; bacteria with specific yellow-green fluorescence were seen clearly, while soil particles (gray areas) fluoresced dull orange-brown.

Large clumps that appear light colored in the upper center of both c and d in Fig. 1 were actually dull orange, and bacterial cells could be distinguished on the margins of these clumps. There is a further improvement in the microscopic appearance of the preparations in the background of fields devoid of soil particles (Fig. 1c, upper left). Such areas were nearly black with no indication that either conjugate was retained by soil films. Perhaps some films wash free as a result of the gelatin treatment.

Results of treatment with conjugates of varying ratios of dye to gelatin are summarized in Table 1. Amounts of rhodamine isothiocyanate ranging from 0 to 120 μg per milligram of gelatin were added to a constant amount of 2 percent autoclaved gelatin, *p*H 10. Conjugated fractions were analyzed on a Klett colorimeter (green filter) for dye content after gel filtration. The standard curve was based on concentrations of RhITC at 0.4 to 40.0 mg per 100 ml in 2 percent gelatin at *p*H 10. Protein content of each conjugated fraction was determined by the Kjeldahl method (*5*). Staining properties were checked on slides recovered from sterilized soil inoculated with *R. japonicum*, and the various gelatin preparations were overlaid with the same batch of *R. japonicum* FITC conjugate. The unconjugated (zero rhodamine) gelatin was prepared with dialysis instead of gel filtration. This preparation may be especially useful for detecting a certain microorganism in the soil without reference to its relations with soil particles. The background was very dark, and specifically stained bacteria could be seen clearly, but soil particles were barely discernible. Mixtures with less than 20 μg of RhITC per milligram of gelatin were generally most satisfactory. Conjugates made with 8 μg of gelatin were used routinely.

The effectiveness of the gelatin-RhITC conjugate in blocking nonspecific adsorption was dependent on the *p*H of the gelatin at conjugation. There was conjugation at *p*H 3, 5, 7, and 8.5, with best conjugation at *p*H 7.0; none of these conjugates prevented nonspecific staining. The only effective gelatin-RhITC conjugates were those which reacted at *p*H 10.

Slides prepared with gelatin-RhITC conjugate were suitable for FA examination for at least 1 week. Longer periods of storage resulted in changes in the fluorescence of the bacteria from yellow-green to orange-yellow. Similar effects were noted when specific conjugate was not added to the gelatin film within a few hours after drying.

Conjugates of gelatin and lissamine rhodamine B were as satisfactory as the gelatin-RhITC conjugates. Lissamine rhodamine B is cheaper but must be converted to the sulfonyl chloride (*7*) before being conjugated. Conjugates prepared with tetramethyl rhodamine isothiocyanate were unsatisfactory over a range of ratios of dye to protein. The red fluorescence of the background was too intense to provide contrast.

Only exploratory studies on the possible use of gelatin preparations for clinical applications were made. Results suggest that gelatin treatment probably can be useful in controlling nonspecific staining for at least some diagnostic problems. Smears of homogenized normal mouse spleen, liver, heart, or lung tissues, impression slides of human spleen, and frozen sections of human lung adsorbed the gelatin-RhITC conjugate, resulting in orange-brown background fluorescence. Nonspecific adsorption of *Rhizobium*-FITC conjugated antiserum was minimum when added to the gelatin-treated preparations. Without gelatin treatment each tissue demonstrated substantial nonspecific staining by the FITC conjugate. Similar tests with deparaffinized sections of human liver and smears of human bone marrow, however, demonstrated little or no decrease in nonspecific staining as a result of gelatin-RhITC treatment.

In further tests (*8*), frozen impression smears of liver and spleen from mice experimentally infected with *Pasteurella tularensis* took up the gelatin-RhITC both in and around tissue cells. Specific staining with *P. tularensis*-FITC conjugate (1 : 8 dilution) was not impaired by the gelatin treatment. Treatment of impressions of infected mouse spleen with either undiluted specific FITC conjugate or undiluted FITC conjugate from normal rabbit resulted in pronounced nonspecific staining. Virtually all of this nonspecific staining was blocked when the undiluted FITC conjugates were applied after gelatin-RhITC treatment. The *P. tularensis* cells showed 4 + fluorescence with the specific staining system despite gelatin treatment and could be seen more clearly and distinctly than on corresponding slides without gelatin. Equally marked benefits were obtained from gelatin-RhITC treatment of paraffin sections of human lymph nodes (from a patient infected with *P. tularensis*). Nonspecific staining was not blocked by gelatin treatment in tests with monolayers of KB mammalian cells (*2*), or with leukocytes in buffy-coat preparations.

The gelatin preparations tested on animal tissues were those developed for use on soil. These preparations provided excellent control of nonspecific staining for soil materials and appeared promising for various tissue preparations as well.

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MN Blood-Group Locus: Data Concerning the Possible Chromosomal Location

Abstract. *Combined data derived from clinical, cytogenetic, and blood-grouping studies of one family suggest that the* MN *locus is on the long arm of either the No. 2 or the No. 4 chromosome.*

Data accumulated during the investigation of a family brought to our attention through a developmentally abnormal child are briefly summarized here as a contribution toward the genetic mapping of the human chromosomes (*1*).