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Further Standardization of the Agglutinin-absorption Test in the Serology of Leptospires *

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Four factors, suspected of influencing the final results of agglutinin-absorption tests used in the diagnosis and classification of leptospires, were investigated in comparative studies: (1) the time required for adequate absorption, (2) the quantitative relationship between antibody titres and amounts of antigen needed for absorption, (3) the possible effect of the Danysz phenomenon, and (4) the absorptive potency of live and formol-treated antigen.

It was found that a 90-minute absorption time was adequate and that with increasing amounts of antigen, titres were continuously reduced, indicating a certain degree of non-specific absorption. The Danysz phenomenon was found to occur in leptospiral serology and the addition of antigen to serum in 3 equal parts at 10-minute intervals is recommended.

The titres of sera absorbed with formol-treated antigen were often found to be lower than titres of sera absorbed with the same amounts of live antigen and some damaging effect of formol on antibody is suspected.

The absorption test of Castellani was first applied to the diagnosis of leptospirosis by Ruys & Schüffner (1934). Subsequently, Borg-Petersen (1938, 1944) proved, in pioneer studies of some strains of the Icterohaemorrhagiae and Hebdomadis serogroups of *Leptospira*, the possibility of revealing by this test serological differences in strains not sufficiently distinguishable in simple cross-agglutination procedures. The importance of the absorption test for diagnosis, epidemiology and classification of leptospires led more laboratories throughout the world to adopt this technique and to develop it in modifications differing more or less from the original procedure. For example, Wolff (1954) performed the absorption test on immune sera diluted to a titre of 1 : 3000, absorbing them with formol-killed, concentrated antigens. Babudieri & Mateew (1961) used a technique differing mainly in the way serum and antigen were mixed. Alexander et al. (1955) reported on repeated absorptions and 4-fold serum dilutions. At the National Communicable Disease Center, Atlanta, Ga., USA, a slightly modified version of Wolff's (1959) technique, recommended by Alexander et al. (1955), was adopted, whereas at the Institute of Epidemiology, Komensky University, Bratislava, Czechoslovakia, a technique was developed that was based on the use of pooled, undiluted immune sera with titres of 1 : 12 800 or 1 : 25 600 absorbed with live concentrated antigens (Galton et al., 1962; Kmety, 1967). None of the above-mentioned methods determined with any greater precision the exact amounts of antigen which should be used for absorption.

Some discrepancies in the typing of strains encountered in recent years explain the growing demand for the absorption technique to be standardized as far as possible in order to ensure reproducibility of results in different laboratories. The absorption test is now the basic and essential procedure for the definitive identification of leptospiral serotypes and is thus the basis on which the present classification system of leptospires is founded. This study attempted to resolve the problem by com-

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parative investigations of the four following factors which may significantly influence the final results of the test: (1) the time required to obtain sufficient absorption; (2) the quantitative relationships between antibody titre and amount of absorbing antigen; (3) the possible influence of the Danysz phenomenon; and (4) the absorptive potency of live and formol-treated antigen.

MATERIALS AND METHODS

Pools of rabbits' sera (3 animals) preserved with 0.5% phenol were used in most of the tests while single-rabbit sera preserved with equal amounts of glycerol were used in the remaining tests.

Reference strains of the Bataviae and Autumnalis serogroups, and in one case the Icterohaemorrhagiae serogroup, were used (*Bull. Wld Hlth Org.*, 1965). In studies of the Bataviae serogroup, a new and recently described serotype, *L. argentiniensis*, was included (Szyfres et al., 1967). The majority of the tests on the Bataviae group were performed at the National Communicable Disease Center with antigens grown in Stuart's or Ellinghausen's media. The antigens were nephelometrically measured before use in agglutination tests and, if necessary, adjusted to a standard of 25–30 nephelos according to Roessler & Brewer (1967). For tests carried out in Bratislava, strains grown in modified Korthof's medium and selected both by eye and by microscopic examination were used (Kmety, 1957).

For all agglutination tests, equal volumes of living antigen suspensions and diluted serum were used, beginning with a serum dilution of 1 : 100 and proceeding in a 2-fold series. After the mixture had been left to stand for about 2 hours at room temperature (20°C–25°C), the result was read according to the 50% agglutination criterion; readings were made at first by two persons independently. However, when differences in the end-points as determined exceeded 1 dilution on few occasions only, the double readings were considered to be unnecessary and were discontinued. Nevertheless, for consistency, the results obtained by only one of these observers are quoted.

Antigens for absorption tests were cultured in 200-ml or 500-ml prescription bottles. Well-grown cultures, mostly between 5 and 10 days old, were spun at the equivalent of about 5000 *g* for 25–30 minutes in a high-speed, angle-headed centrifuge and concentrated about 40–80 times. The killed antigen was prepared by the addition of a 10% formol-buffered saline solution to a well-grown culture to a final concentration of 0.25% of formol. The formolized culture was left to stand for 3 hours at room temperature (20°C–25°C) before centrifugation. To ensure that the same amounts of antigen were used in comparative studies, the technique was modified so that the concentrated living antigen was at first adjusted to McFarland Standard No. 10 (see Helper, 1953) by the addition of saline (McFarland, 1907) and the antigen was then divided into 2 equal parts, one of which was treated with formol as previously described. The concentrated and adjusted antigen, living or killed, was added to the undiluted serum in 2 or 3 equal parts at 10-minute intervals, usually in a ratio of 24 (i.e., 3 × 8) parts of antigen to 1 part of serum. After each addition of antigen, the mixture was shaken and allowed to stand at room temperature (20°–25°C) for 90 minutes, unless it is stated otherwise. The antigen was then removed by centrifugation and the supernatant (absorbed serum) was stored at −20°C, if it was not to be tested immediately.

In assays of the Danysz phenomenon, the serum was first diluted with saline (1 part of whole serum or 2 parts of glycerolated serum being added to 25 parts or 24 parts of saline, respectively. The diluted serum was then added in 3 approximately equal parts (9 + 9 + 8) at 10-minute intervals to 24 parts of antigen if it is not stated otherwise (Fig. 3, p). Again, the antigen–serum mixture was centrifuged and the supernatant was stored at −20°C if it was not to be tested at once.

RESULTS

Time relationship in absorption

In the first series of absorption tests, a comparative study was performed to show whether overnight storage for 18 hours of the serum–antigen mixture, as practised in most laboratories, results in better absorption than when the serum is exposed to antigen for a shorter period.

Results of parallel tests were compared; in 5 tests with live antigens and in 2 with killed antigens, the serum being exposed to antigen for 18 hours and for 90 minutes, respectively. In 3 additional tests with live antigen, the time of absorption was shortened to 60 minutes.

The results of these tests (Fig. 1) revealed clearly that there was no significant difference in titre in any of the 10 investigated pairs of sera, whether absorbed with live or formol-treated antigen. It may be assumed, therefore, that a period of 90 minutes, and apparently also of 60 minutes, is sufficient to ensure

FIG. 1

RESULTS OF COMPARATIVE ABSORPTION TESTS PERFORMED AFTER SPECIFIED PERIODS OF ABSORPTION WITH LIVE AND KILLED LEPTOSPIRAL ANTIGENS

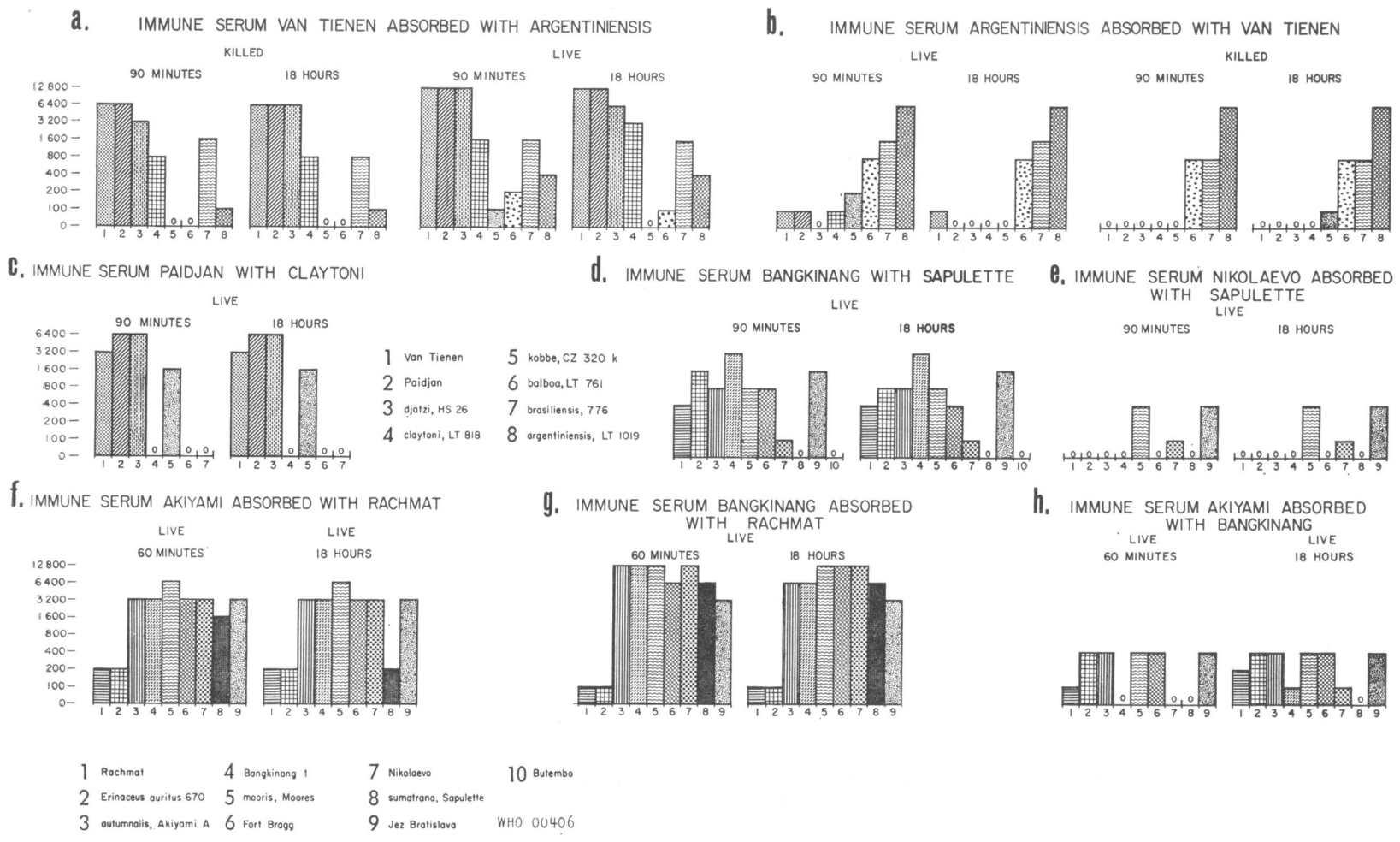


FIG. 2

RESULTS OF COMPARATIVE ABSORPTION TESTS PERFORMED WITH VARYING AMOUNTS OF LIVE OR KILLED ANTIGENS

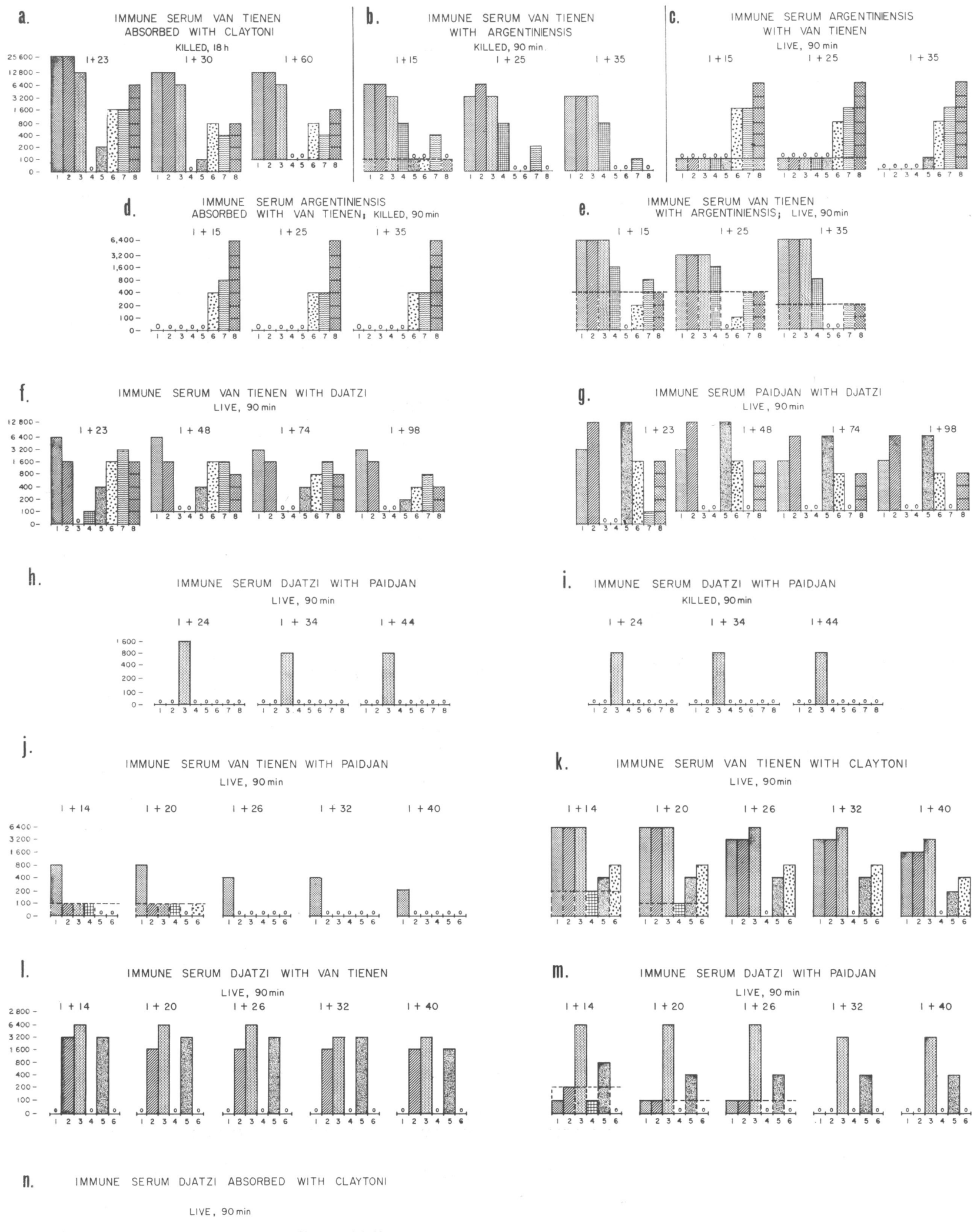


FIG. 3

RESULTS OF COMPARATIVE TESTS ON THE OCCURRENCE OF THE DANYSZ PHENOMENON IN LEPTOSPIRAL ABSORPTION TESTS

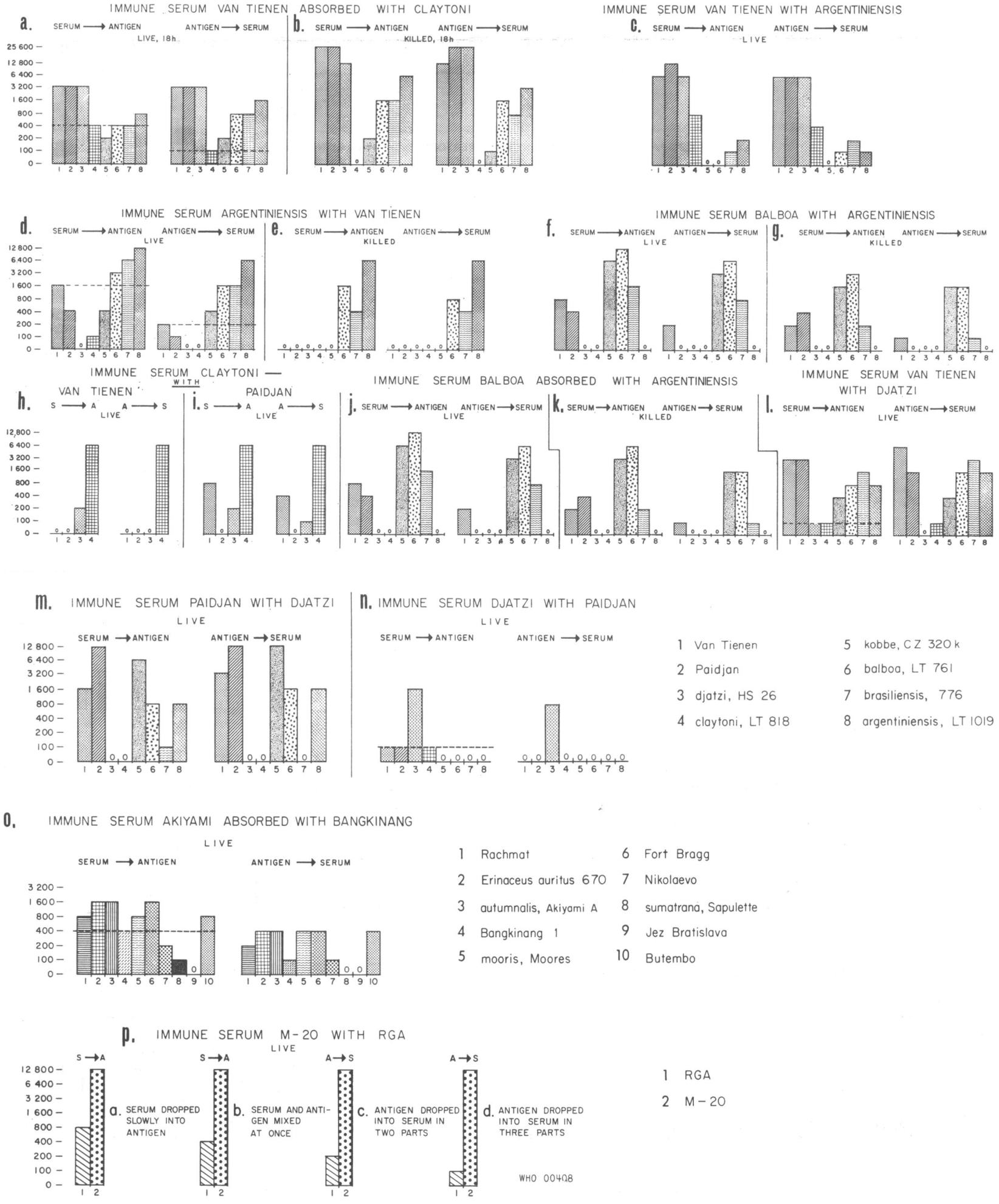
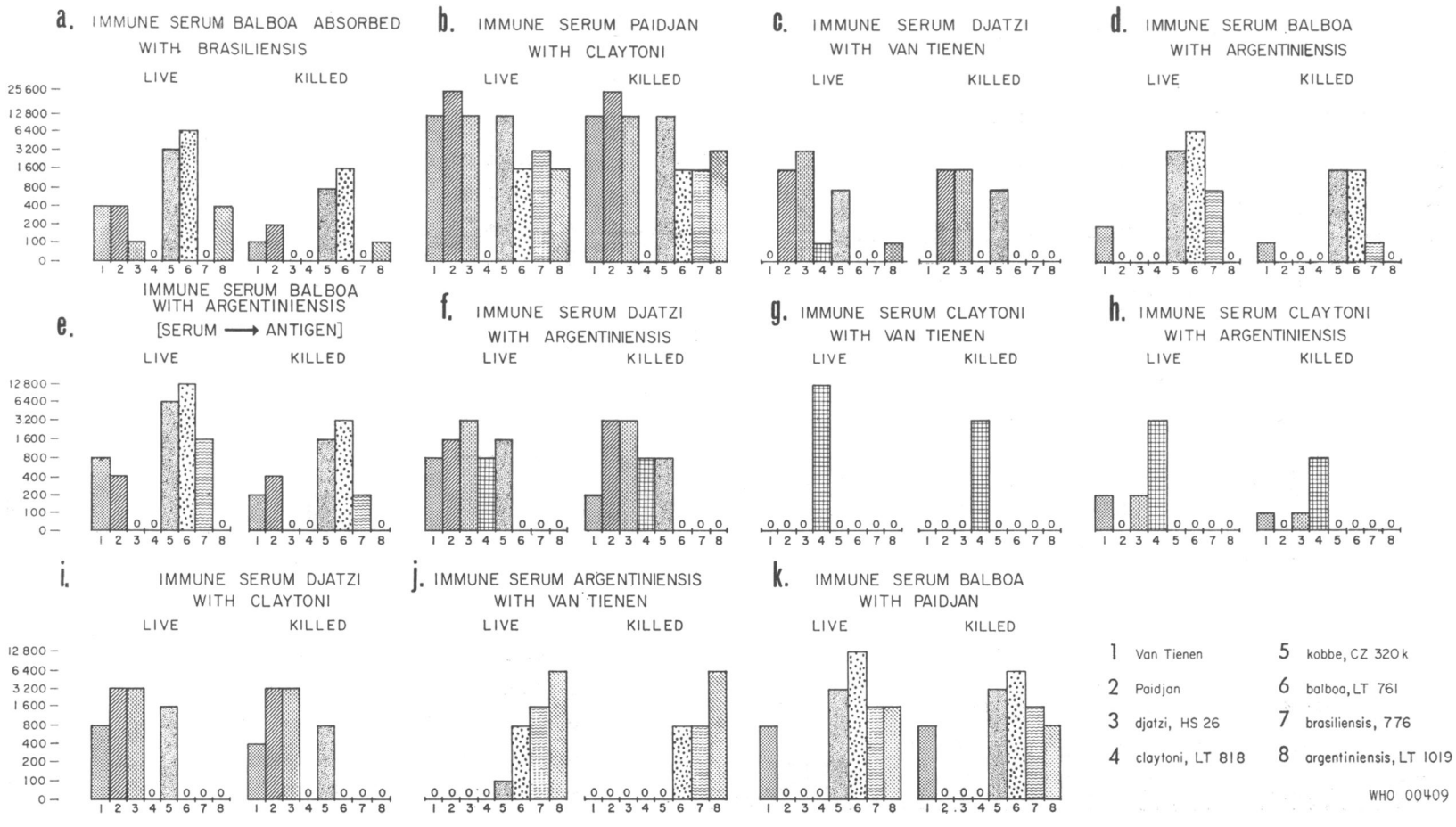


FIG. 4

RESULTS OF COMPARATIVE ABSORPTION TESTS WITH LIVE AND KILLED LEPTOSPIRAL ANTIGEN



the expected binding of antibodies to the corresponding sites of antigen. Thus, the time to be allowed for absorption procedures requires no standardization. From a practical point of view, however, an absorption time of 90 minutes is suggested.

Quantitative relationships between serum titre and amount of antigen

The amount of antigen necessary for the complete absorption of antibodies detectable by the agglutination test is a matter of great importance because antigen used in too small amounts is not able to remove all corresponding antibodies, and in too large amounts it is believed to cause non-specific absorption (Kmety, 1967).

To obtain a better insight into the quantitative relationship between antibody, as expressed by the titre, and the amounts of antigen required for absorption, 17 tests (including 4 with killed antigen) were performed, using increasing amounts of antigen. The smallest proportion was 1 part of serum to 14 parts of antigen; the largest was 1 part of serum to 98 parts of antigen. The proportions used in the tests are shown in Fig. 2.

The results of these investigations show with considerable consistency that increasing the amounts of antigen caused a continuous decrease in titre. From the following tabulation it can be seen that if the titres in tests performed with the smallest and the largest amounts of antigens are compared, the titre was lower in more than 4 out of 5 of the latter tests, and that there was no change in titre in fewer than 1 out of 5.

Titre *	*No. of reactions*	
Same	17	
1 lower	53	77 (82%)
2 lower	22	
3 lower	2	
Total	94	

* Of serum absorbed with large amounts of antigen, in comparison with the titre of serum absorbed with small amounts of antigen.

It was noted that even 4-fold amounts of antigen (Fig. 2, f and g) caused, at the most, a difference of 2 titre steps. However, even if the sera absorbed with large amounts of antigen did not show a substantial decrease in titre, the agglutination was much weaker in the highest dilution; in fact, the reaction was sometimes on the borderline of positivity.

The results presented in Fig. 2 also allow an estimation to be made of the amounts of antigen required to achieve adequate absorption. In most cases, the ratio of 1 part of serum to 24 parts of concentrated antigen of a density corresponding to McFarland Standard No. 10 is sufficient to give adequate absorption of the antibodies present in a titre of 1 : 12 800. If the titre of antibodies to be absorbed is lower than 1 : 12 800 (for instance, about 1 : 3 200), satisfactory absorption can be obtained with smaller amounts of antigen as indicated in Fig. 2, tests b, c, d, j, l, n, o and p.

It must be admitted that the evaluation of the antigen density by eye, in comparison with an adjustment to the McFarland Standard, may introduce a considerable error. However, the above-mentioned results show that even a 2-fold increase in the amount of antigen does not greatly change the residual titres. Therefore, an exact nephelometric measurement of antigen density for absorption procedures does not seem to be absolutely necessary.

In antigen–antibody relationships, there seems to be a certain difference between antibodies corresponding to antigens in the absorbing strain (antibodies to be absorbed), and antibodies not corresponding to antigens in the absorbing strain (antibodies which should remain in the absorbed serum), as is clearly shown in the results of tests b, e, j, k, m, n, p and q in Fig. 2. These tests indicate that even slightly increased (less than double) amounts of antigen are able to reduce the residual titre against the absorbing strain by 1 step, whereas considerably larger amounts of antigen are usually necessary to cause a titre reduction of the non-corresponding antibodies.

The quantitative antigen–antibody relationships are apparently more critical as far as antibodies corresponding to the absorbing strain are concerned. Both effects must be taken into account in order to increase the reliability of the absorption test.

The Danysz phenomenon

Danysz (1902) described the observation that mixtures of the same amounts of toxin and antitoxin resulted in different toxicities, depending on the way in which the two components were mixed. If toxin was added drop by drop to antitoxin, the final mixture remained toxic. In contrast, if antitoxin was dropped into toxin, free, unbound antitoxin was found in the final mixture. This phenomenon was later explained by Heidelberger & Kendall (1929) as the result of the different proportions in which antigens and antibodies are able to combine.

It was thought that the Danysz phenomenon might occur in the absorption procedures with

leptospires. In order to study this possibility, 16 comparative tests, including 4 tests with killed antigens, were performed.

The results presented in Fig. 3 (mainly in tests a, c, d, e and o) show clearly that the same amounts of antigen, when dropped into serum, give better absorptive effects than when serum is added to antigen. The difference in titre is equal to 1 or 2 steps. An excellent example is Fig. 3, p, which shows that the addition of antigen to serum in 2 or 3 equal parts at 10-minute intervals improves the final absorption, reducing the residual titre against the absorbing strain by 2 or 3 steps, respectively.

In some tests (Fig. 3, f, g, j, k and n), where apparently the amounts of antigen used for absorption were too large, the titre of the residual antibodies also was lower in those mixtures in which antigen was added to serum. This must be explained as a consequence of non-specific absorption in tests that quantitatively were not well balanced, as discussed in the previous section.

Evidently, the Danysz phenomenon applies also to the serology of leptospires. Thus the technique of agglutinin-absorption by the addition of antigen to serum in 2 or 3 equal parts at intervals of about 10 minutes seems to be appropriate.

Absorption with live and formol-treated antigens

At the present time, absorption, whether performed with formol-treated or with live, concentrated antigen, seems to give similar results; however, comparative tests giving exact results have not yet been reported. To evaluate both procedures, a series of 14 comparative tests were performed. Exactly the same amounts of antigen for absorption and the same live cultures for titration of the absorbed sera were used. No substantial differences were seen in the results obtained in these tests (Fig. 4 and Fig. 2, h and i). However, sera absorbed with killed antigen showed a lower titre in nearly 2 out of every 3 tests.

The differences in titre in the comparative absorption tests are summarized in the following tabulation:

Titre *	*No. of tests run*	
1 higher	2	
Same	19	
1 lower	18	31 (60%)
2 lower	11	
3 lower	2	
Total	52	

* Of serum absorbed with killed antigen, in comparison with the titre of serum absorbed with live antigen.

In 52 cases, corresponding residual titres could be compared; in 19 the titres did not differ, but in 33 they differed by 1 or 2, or occasionally 3, titre steps. However, while the residual titre was higher in the serum absorbed with formol-treated antigen in only 2 cases, it was higher in the serum absorbed with live antigen in 31. Since the same amounts of formol-treated antigen and live antigen were used, and since the assumption of a higher absorbing potency of formol-treated antigen is considered to be unreasonable, some damaging effect of formol on the antibodies must be taken into account.

In order to approach this hypothesis, the results of agglutination tests performed with 13 sera of the Bataviae serogroup treated with formol in the same concentration as that used in the absorption procedure were compared with the results of agglutination tests performed with untreated sera. In 77 tests no difference in titre was observed and in 16 tests the titre of the formolized serum was lower by 1–2 steps; but in 11 tests a higher titre was found. It must be admitted, however, that the formol generally acted on the unabsorbed sera for a much shorter time than it did on the absorbed sera. The absorbed sera had, in most cases, been stored at −20°C for several weeks before being tested, and the results may have been influenced also by the effects of this storage.

The question why sera absorbed with formol-treated antigen gave mostly lower titres remains open, but some damage to antibodies by the formol is suspected.

DISCUSSION

The agglutinin-absorption test is a fundamental procedure in the current classification of leptospires which is based mainly on serological properties of the strains. There is a general need to establish a standard technique in order to ensure the reproducibility of results and to avoid discrepancies between the results of different laboratories.

The present study shows that certain quantitative relationships between antibodies and antigens must be carefully considered and that the ability of antibodies to combine with antigens in different ratios, depending on the way in which the antibodies and antigens are mixed, must be respected. The use of formol-treated antigens is not recommended because the treatment may result in damage to the antibodies. All these factors may cause some variability in the results. However, even in well-balanced absorption tests, there remain some other factors, such as the immune serum, the agglutinability of strains, tech-

nical precision, etc., which may possibly affect the perfect conformity of results. These other possible causes of variability probably cannot be eliminated completely.

The criterion for differentiating serotypes contains the statement that " adequate amounts " of antigen should be used in absorption tests, but there is no specification of how much antigen should be considered adequate (*Bull. Wld Hlth Org.*, 1965). Wolff & Broom (1954) considered complete absorption to be achieved when no agglutinin against the absorbing strain could be demonstrated in a serum dilution of 1 : 30 which had had a homologous titre of 1 : 3000 before absorption. The possibility of non-specific absorption caused by excessive amounts of absorbing antigen was not considered. Nevertheless, the results reported in the present paper indicate that the use of absorbing antigens in excessive amounts does cause some degree of non-specific absorption. The problem of " adequacy " is apparently a question of the minimum amount of antigen required to achieve appropriate absorption of the corresponding antibodies.

The use of antigen of an adjusted density in suitable proportions makes a substantial contribution to the standardization of the test but the proof that the absorption test was not performed with an excess of antigen is given only when the first dilution of the absorbed serum still reacts with the absorbing strain. This requirement is very strongly recommended in cases where the required 10% post-absorption titre limit is just reached, and in cases where the differences between two strains are below the 10% limit. In such cases, precision is of particular importance because a difference of only 1 titre step is decisive for the recognition of a new serotype. When the homologous titre, i.e., the titre with the strain used for immunization, is not significantly reduced after absorption, and the 10% titre limit is evidently exceeded, a negative reaction with the absorbing strain in the first dilution of the absorbed serum may also be considered to reflect a well-balanced test.

The acceptance of this recommendation, as well as the fact that a variability of +1 or −1 titre step is within the limitations of the technique itself, indicates that a serum with a high titre should be used. An immune serum with a titre of 1 : 12 800 or 1 : 25 600 is considered optimal for use in an agglutinin-absorption test for purposes of classification.

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RÉSUMÉ

NOUVELLE NORMALISATION DE L'ÉPREUVE D'ABSORPTION DES AGGLUTININES DANS LA SÉROLOGIE DE LA LEPTOSPIROSE

Par une série d'essais comparatifs, on a recherché dans quelle mesure certains facteurs peuvent modifier les résultats des épreuves d'absorption des agglutinines servant au diagnostic de la leptospirose et à la classification des souches de *Leptospira*. Ont été étudiés: *a*) le laps de temps requis pour obtenir une absorption suffisante; *b*) les relations quantitatives entre les titres d'anticorps et les quantités d'antigène nécessaires à l'absorption; *c*) le rôle éventuel d'un phénomène de Danysz; *d*) la capacité d'absorption des antigènes vivants et des antigènes tués par le formol.

D'épreuves effectuées avec des antigènes vivants ou tués, il ressort que l'absorption n'est pas sensiblement améliorée par un contact de l'antigène et des anticorps prolongé pendant 18 heures. Du point de vue pratique, on peut admettre que la liaison antigène–anticorps est réalisée après 90 minutes de contact. Si l'on utilise une trop grande quantité d'antigène, on constate régulièrement une diminution du titre des anticorps sériques, ce qui dénote un certain degré d'absorption non spécifique.

On a mis en évidence l'existence d'un phénomène de Danysz: pour une même quantité d'antigène, l'absorption est plus effective lorsque l'antigène est incorporé au sérum en petites quantités successives que lorsqu'on ajoute le sérum à l'antigène. La meilleure technique, semble-t-il, consiste à ajouter l'antigène au sérum en deux ou trois fractions aliquotes à intervalle d'environ 10 minutes. Des épreuves comparatives indiquent que les sérums absorbés par un antigène formolé présentent des titres inférieurs à ceux des sérums absorbés par des antigènes non traités.

Les implications de ces données en ce qui concerne la méthodologie des épreuves d'absorption et les critères à utiliser pour la classification des leptospires sont examinées.

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