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Attempts to Influence Antibody Formation by Tetracycline

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Clinical and experimental findings by a number of authors (Weinstein & Tsao, 1946; Rantz, Boisvert & Spink, 1946; Harrison, 1946; Skinses & Woolridge, 1948; Loge & Kilbourne, 1948; Jordan, 1949; Weinstein, Bachrach & Perrin, 1949; Schwartzman, 1950), providing evidence that treatment of infectious processes with massive doses of antibiotics inhibits humoral immunity mechanisms, were summed up by Green, Wohl and Waife (1951) and Stevens (1953). In such cases, inadequate immunity is due to rapid suppression of the causative agent and its prompt elimination from the body of the host. The depressive effect of antibiotics on antibody formation has also been studied as a general immunological problem, however. One team of workers investigated the protective effect of antibiotics against the action of bacterial endotoxins (Miller & Boor, 1946; Miller, Hawk & Boor, 1948; Spink, 1954, 1956). Other authors studied inhibition of antibody formation by antibiotics in immunization with widely different types of antigens (Marchal, Nicot & Fery, 1949; Green, Wohl & Waife, 1951; Slanetz, 1953, 1954; Carpenter *et al.* 1955; Averyanova, 1957; Chumachenko, 1957a, b; Uraleva, 1958; Kokushkina & Yegorova, 1959; Toshkov, Sheikova & Zachariev, 1959; Toshkov, Sheikova & Boyadzhiyeva, 1959). In a series of experiments, Lamensans and Farhi (1955, 1956), Farhi (1957a b), and Farhi and Lamensans (1955, 1956a, b) who studied the inhibitory effect of chloramphenicol on antibody formation and lowering of the defences of the organism after the administration of antibiotics, also determined modifications in the antigenic structure of the bacterial agent as a result of the effect of the antibiotic.

In the present work the authors investigated the effect of different doses of tetracycline on antibody formation, using several different types of antigen: (a) inactivated corpuscular Brucella antigen, emulsified in liquid paraffin accordilnl to Freund's principle, and plain Bruceag bacterin; (b) active influenza virus emulsified in peanut oil; (c) killed *Coxiella burneti* antigen emulsified in liquid paraffin.

In the final series of experiments, the formation of neutralizing antibodies was studied in rabbits infected subcutaneously with Herpes simplex virus.

MATERIALS AND METHODS

Rabbits of several strains, weighing 2.5—3 kg., were used. They were divided into groups of ten, according to sex. The *Coxiella burneti* immunization experiment was carried out in groups of twelve male guinea pigs weighing 550 g. Before the experiment all the animals were tested to ensure that their serum contained no antibodies against the given antigens.

In the experiment in which Brucella antigen was used, strain Br O of *Brucella abortus* was inactivated for one hour at 58° C, using a 48-hour culture grown on Huddleson's agar. Standard bacterial suspensions were obtained by measuring on a Hilger absorptiometer. Inoculum for long-acting immunization was prepared by emulsification of the Brucella antigen in liquid paraffin, using lanolin as

emulsifier (three parts liquid paraffin, one part lanolin, two parts antigen). Bacterin and antigen, with lipoid adjuvant, were administered intramuscularly (John & Schindler, 1958). Long-acting inoculum was administered in amounts of 1 ml. into the muscles of the right hind limb.

Blood for determining antibodies by agglutination and the complement-fixation reaction was usually collected at weekly intervals by cardiac puncture. Both reactions were carried out by standard laboratory methods (John, 1958). The geometric means of the individual serological results were computed according to the formula: geometrical mean = antilog $\frac{\Sigma(fl)}{n}$, in which f is the frequency of the reciprocal value of the titre, l is the logarithm of the reciprocal value of the titre and n is the number of animals in the group examined.

A skin allergy test was carried out with the following antigens: (a) Brucella bacterin; (b) the supernatant fluid obtained by centrifugation (12,000 r.p.m. for 30 minutes) of a Brucella suspension in physiological saline, shaken with glass beads for 60 minutes; (c) non-antigenic allergen F (Bioveta, Nitra). All three types of antigen were injected intradermally in 0.1 ml. physiological saline. The degree of allergic reaction was evaluated according to the scheme of Römer and Joseph, as interpreted by Metaxas (Metaxas & Metaxas-Buehler 1949, 1954, 1955): +++— "cocarde" reaction with livid centre, pallid intermediate zone and erythematous periphery; marked induration, reaction lasting for several days, usually followed by necrosis; ++ — pale papule, with erythema and mild induration; + — erythema and mild oedema, reaction not lasting longer than 48 hours; ± — signs of erythema.

For immunization with influenza virus, living strain A_2 virus was used purified by Takátsy's method by reducing the NaCl ion concentration. The virus was emulsified in peanut oil, using 4% aluminium stearate as the emulsifier, in the ratio of one part peanut oil with aluminium stearate to one part purified virus in physiological saline (Patočka *et al.* 1958).

The inoculation dose of 1 ml. emulsion, containing 800 haemagglutination units virus, was injected intramuscularly. Sera were collected from the rabbits by cardiac puncture at regular intervals and were examined for HI antibodies and complement-fixing antibodies. A neutralization experiment was carried out with sera collected six weeks after immunization. In this experiment, 100 ID_{50} of the virus was mixed gradually with double the serum dilution. After 24 hours' incubation in a refrigerator at 4° C, the virus was injected into the allantois of chick embryos. The dilution at which the virus could no longer be demonstrated in the allantoic fluid by the haemagglutination test was taken as the neutralization titre of the serum.

Guinea pigs were immunized with killed *Coxiella burneti* antigen (Bioveta) containing four CF units/0.1 ml. The antigen was emulsified in liquid paraffin, together with lanolin, in the same way as for Brucella antigen. The inoculation dose for each animal, which was administered intramuscularly into the right hind limb, was 0.4 ml., containing 10 CF units. Sera were collected by cardiac puncture three and five weeks after immunization and were examined by fixation of complement and by microagglutination on a slide.

In the herpes simplex experiment, a group of twenty rabbits was infected by the intracorneal administration of a 10% brain suspension from mice infected with the laboratory strain HC herpes simplex, and the interval from the development of keratitis to encephalitis was observed.

Another group of twenty rabbits was infected subcutaneously with 1 ml. mouse brain suspension containing herpes virus (strain HC, LD_{50} for mice on intracerebral administration $10^{-3.9}$). After 28 days

blood was collected (from the experimental and the control group) by cardiac puncture. The sera of five rabbits from the same group were always pooled in equal proportion, giving two pooled sera from each group for the neutralization reaction. The neutralization test was done in white mice weighing 20 g. Equal amounts of serum were mixed with decimal dilutions of brain suspension from mice infected with strain HC, to a final dilution of 10^{-7}. The pooled serum, together with different dilutions of brain suspension, was incubated for two hours at laboratory temperature. Amounts of 0.03 ml. of each dilution were then used for the intracerebral infection of groups of five mice. The virus was simultaneously titrated without the presence of neutralizing antibodies.

Tetracycline was always administered to the experimental animal intramuscularly, in the doses given in the results. Administration was always commenced on the same day as immunization or infection. Tetracyklin Bayer was used—first the preparation for intramuscular administration, but in most of the experiments the preparation for intravenous administration (crystalline tetracycline hydrochloride, without other ingredients). The intravenous preparation was also administered intramuscularly and was well tolerated.

Procaine penicillin was administered intramuscularly to one group of rabbits (50,000 units daily).

Cortisone was administered in the form of the intramuscular preparation (Cortisone, Continental Pharma).

The faeces of the experimental animals were regularly examined by aerobic and anaerobic culture; 0.1 g. faeces was diluted with physiological saline and after culturing constant amounts of these dilutions on blood agar and counting the number of colonies, the results were expressed as the number of microorganisms per gramme of faeces.

While administering tetracycline the rabbits were weighed regularly and any increase or loss in weight was recorded.

RESULTS

The groups of rabbits immunized intramuscularly with Brucella antigen emulsified in liquid paraffin (1.5×10^{10} inactivated brucellae) received 25 mg. tetracycline intramuscularly daily per animal

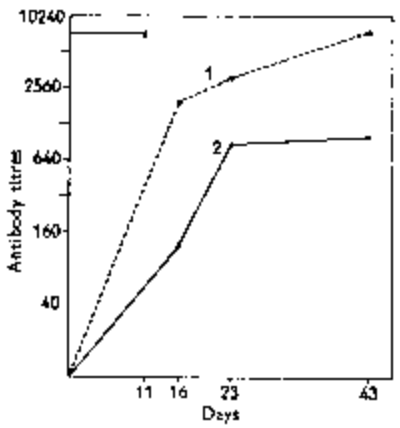

Fig. 1. Geometrical means of agglutinin titres in rabbits immunized with *Brucella abortus* (1.5×10^{10} microorganisms) emulsified in liquid paraffin (curve 1) and in rabbits immunized in the same way, to which 25 mg. tetracycline was administered intramuscularly daily for 11 days (curve 2).

for eleven days from the day of immunization. The relatively low dose of tetracycline in this case markedly reduced the agglutinin level (Fig. 1) and the CF antibody level (Fig. 2) as compared with

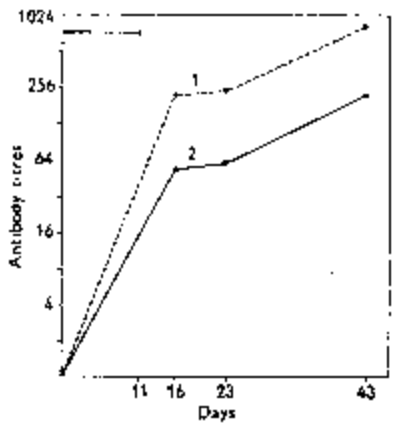

Fig. 2. Geometrical means of complement-fixing antibodies under same experimental conditions as in Fig. 1.

Table 1. Intensity of skin allergy tests in rabbits immunized with corpuscular Brucella antigen emulsified in liquid paraffin. The first group received tetracycline (25 mg. daily i. m.) for 11 days from the day of immunization

Number of inactivated brucellae	Degree of delayed allergic reaction					Number of rabbits
	0	±	+	++	+++	
	Long-acting immunization + tetracycline					
12×10^7	1	4	2	1		8
12×10^6	4	2	2			8
12×10^5	5	3				8
	Long-acting immunization					
12×10^7				7	1	8
12×10^6			5	2	1	8
12×10^5	1	4	2	1		8

Table 2. Intensity of skin allergy tests in rabbits immunized with Brucella suspension. The first group received tetracycline (40 mg. daily i. m.) for 11 days from the day of immunization

Allergen	Degree of delayed allergic reaction					Number of rabbits
	0	±	+	++	+++	
	Single immunization dose of inactivated brucellae + tetracycline					
Inactivated brucellae 12×10^6	7	3				10
Soluble antigen	6	4				10
Allergen F	4	6				10
	Single immunization dose of inactivated brucellae					
Inactivated brucellae 12×10^6	5	1	2			8
Soluble antigen			3	5		8
Allergen F		1	3	4		8

the controls. After discontinuing the antibiotic both types of antibodies relatively soon reached approximately the same levels as in the controls.

During administration of the antibiotic, weight gains were determined in both groups of animals. In the group to which tetracycline was administered, the total weight gain per rabbit on the last (11th) day amounted to 15—25 dkg. (from the outset of the experiment), and was the same as in the controls.

When studying changes in the intestinal flora during administration of the above doses of tetracycline, it was found that after five days' administration of tetracycline, the number of *Escherichia coli* organisms, which in normal rabbits ranges from tens to hundreds of millions, decreased, indeterminately and inconsistently, to a few milion, while after eight to ten days it could be expressed in hundreds of thousands. After eleven days' administration of tetracycline, a

marked reduction in the number of anaerobic bacteroids was found. In general, there were no changes in the numbers of micrococci, enterococci, Gram-positive sporulating aerobic bacteria and the rarer anaerobic Clostridia.

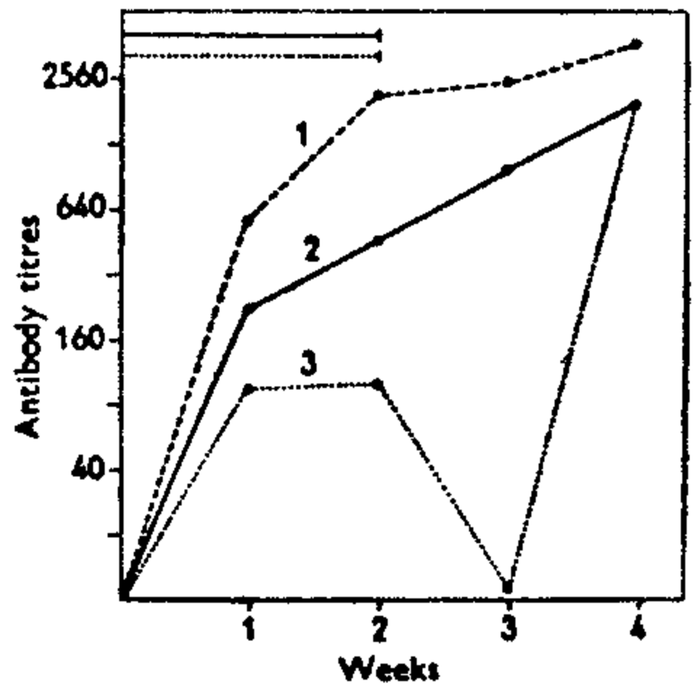

Fig. 3. Geometrical means of agglutinin titres in rabbits subjected to long-acting immunization with *Brucella abortus* (1.5×10^{10} microorganisms) emulsified in liquid paraffin (curve 1), in rabbits immunized in the same way, to which tetracycline (curve 2) was administered for 14 days and in animals to which cortisone was administered 14 days from the day of immunization (curve 3).

In rabbits subjected to long-acting immunization and simultaneously to marked sensitization, the intensity of the delayed allergic reaction after the intradermal injection of Brucella bacterin was studied. The degree of the allergic reaction was evaluated according to the scheme of Römer and Joseph (v. above). The reaction was done four weeks after immunization, i. e. 17 days after discontinuing tetracycline. The administration of tetracycline markedly reduced the intensity of the allergic reaction (Tab. 1). In all eight experimental rabbits, to which tetracycline was administered for eleven days, the degree of sensitization was much less than in the controls.

Under similar experimental conditions, antibody formation was studied during long-acting immunization, using the same inoculum but raising the dose of tetracycline (40 mg. for 14 days). This experiment included an additional group of animals to which 10 mg. cortisone was administered intramuscularly for 14 days. In the group to which tetracycline was administered, agglutinin formation already decreased during the first week after immunization (Fig. 3) and did not approach the level in the immunized

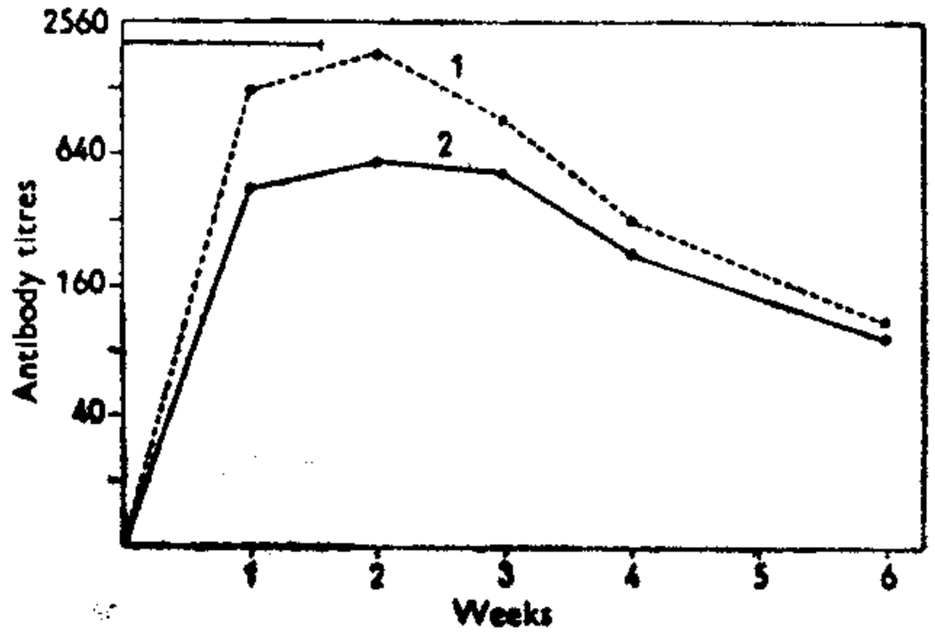

Fig. 4. Geometrical means of agglutinin titres in rabbits immunized with Brucella bacterin (4×10^{10} microrganisms; curve 1). Curve 2 gives the geometrical means of agglutinin titres in animals immunized in the same way, to which tetracycline was administered for 11 days.

controls until the fourth week. Cortisone also markedly reduced antibody formation. This is an interesting finding, since cortisone usually depresses antibody formation only if its administration is commenced several days before immunization (Fagreus, 1952). Its depressive effect was most marked during the third week of the experiment, i.e. a week after administration of the final dose. It is interesting to note the subsequent sharp increase in antibodies during the fourth week, when the long-acting antigen still provided an adequate stimulus for antibody formation after the cortisone screen had disappeared. The effect of cortisone was much stronger than that of tetracycline. Neither substance, however, markedly reduced the production of complement-fixing antibodies under the given experimental conditions.

Relatively poor inhibition of the primary immune response was obtained in rabbits immunized intramuscularly with a single dose of corpuscular Brucella antigen (4×10^{10} microorganisms), to

which 40 mg. tetracycline was administered intramuscularly daily for eleven days (Fig. 4). The geometrial means of the agglutination titres differed only slightly from the average for the control group. There was likewise no discernible difference

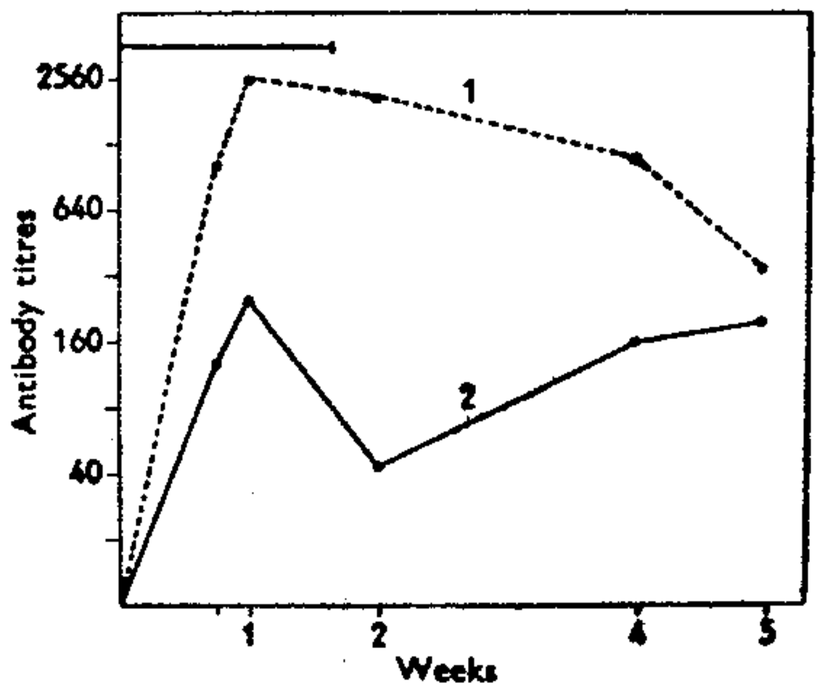

Fig. 5. Geometrical means of agglutinin titres in rabbits immunized with Brucella bacterin (2×10^{10} microorganisms), to which doses of 100 mg. tetracycline were administered (curve 2). Curve 1 shows the geometrical means of agglutinin titres in the control group.

in the primary immune response in the production of complement-fixing antibodies.

In the given experimental arrangement, however, tetracycline depressed the development of hypersensitivity to the three different types of Brucella allergen. The intensity of skin allergy tests carried out four weeks after primary immunization was less marked in rabbits treated with tetracycline than in the controls. In animals not subjected to long-acting immunization and in the controls, sensitivity was naturally less marked than in animals to which an adjuvant was administered.

Depression of antibody formation was most pronounced on raising the daily dose of tetracycline to 100 mg. and administering it for twelve days (Fig. 5). Immunization was carried out intramuscularly with Brucella bacterin, the dose containing 2×10^{10} micro-organisms in 0.5 ml. physiological saline. In this group depression of complement-fixing antibodies was also recorded (Fig. 6). The large dose of antibiotic had a marked effect on the weight of the experimental animals, however. After three days the animals began to lose weight and by the twelfth day their weight had dropped to 87 — 78%

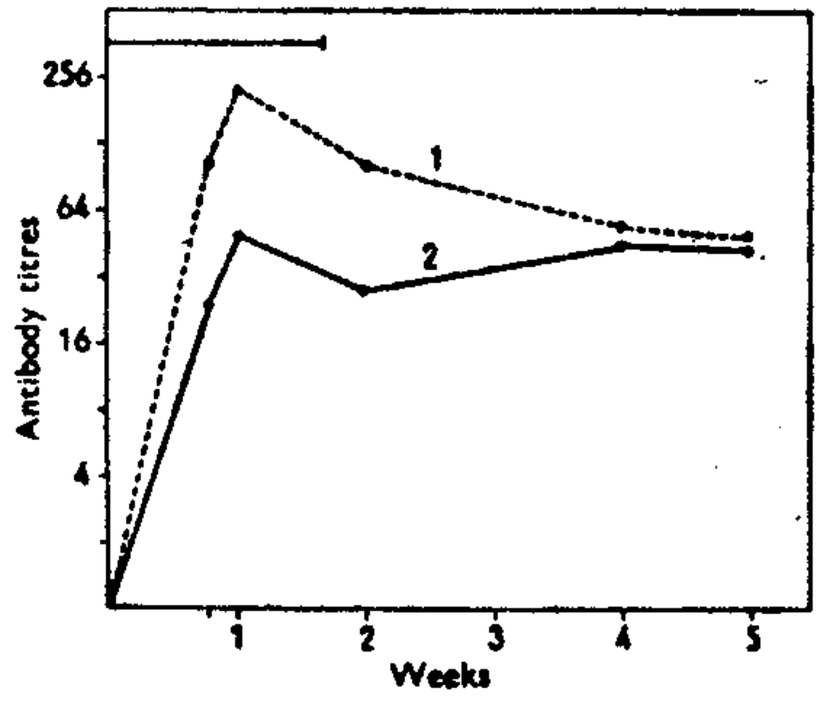

Fig. 6. Geometrical means of complement-fixing antibodies under same experimental conditions as in Fig. 5.

of the original value. Quantitative evaluation of the intestinal flora showed that five days after the administration of 100 mg. doses of tetracycline the number of *Escherichia coli* per gramme faeces could be expressed in tens of thousands and thousands only, and by the 10th to 12th day it was impossible to detect any in some of the animals. Anaerobic bacteroids were significantly inhibited, while no significant changes occurred in the numbers of intestinal micrococci, enterococci, Gram-positive sporulating aerobic bacteria and anaerobic Clostridia.

In another series of experiments, 100 mg. doses of tetracycline were administered for seven days after long-acting immunization of a group of rabbits with living influenza virus (A_2) emulsifiied in peanut oil. Marked depression of the formation of haemagglutination-inhibiting antibodies was observed in rabbits treated with tetracycline (Fig. 7). The appearance of these antibodies in the serum was delayed, their level was very low and after nine weeks fell below significant values, while in the serum of control animals marked HI antibody titres could

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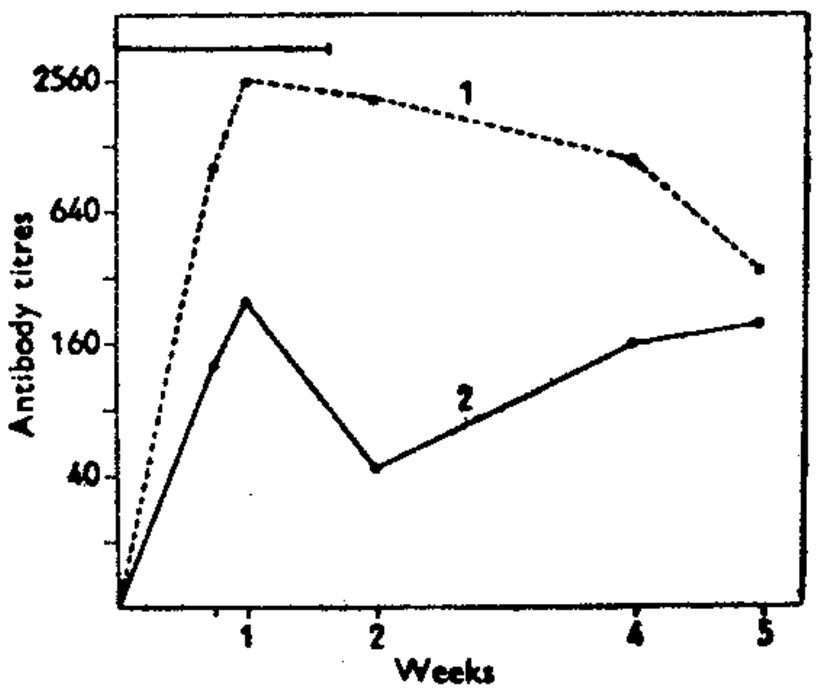

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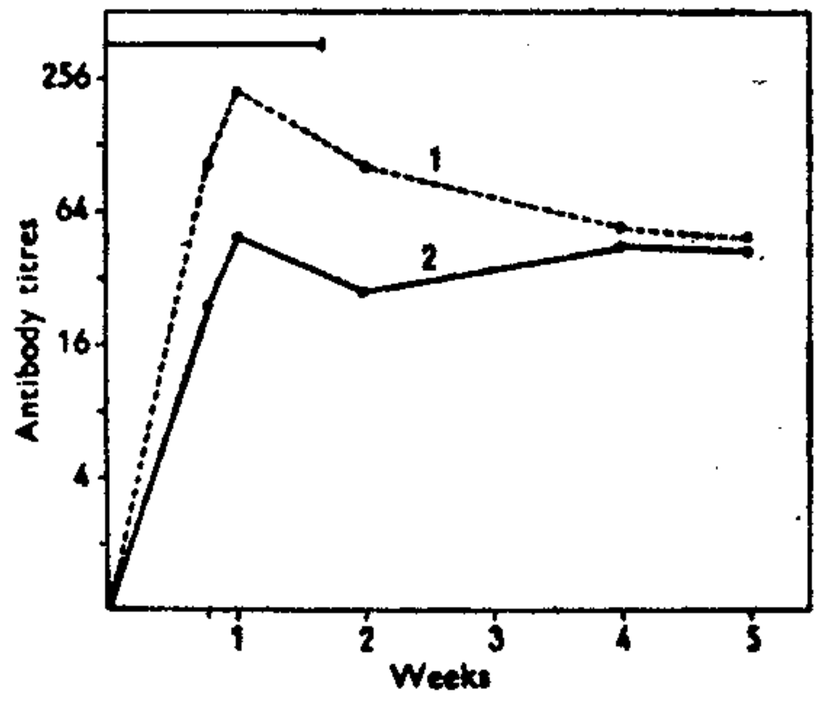

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still be demonstrated six months after immunization. Seven days' intramuscular administration of procaine penicillin (50,000 units daily) produced only a mild decrease in the antibody level. The same applies, to a less marked degree, to complement-fixing antibodies.

Although HI antibodies can be regarded as a sensitive indicator of the immunity of an organism, neutralization tests were carried out with pooled sera collected six weeks after immunization. In animals to which procaine penicillin was administered, neutralizing antibodies attained a titre of 1 : 256—the same as in long-acting immunization without antibiotics. In the group to which tetracycline was administered the titre was 1 : 64.

In an attempt to determine the depressor effect of tetracycline on immunogenesis, using different types of antigen, the antibiotic was administered for ten days from the day of immunization, in amounts of 25 mg/kg. body weight, to a group of guinea pigs subjected to long-acting immunization with inactivated *Coxiella burneti* emulsified in liquid paraffin. In this experiment relatively slight depression of the formation of complement-fixing antibodies was determined three to five weeks after immunization (Fig. 8). A similar relationship applies to agglutinating antibodies, in which depression was more pronounced.

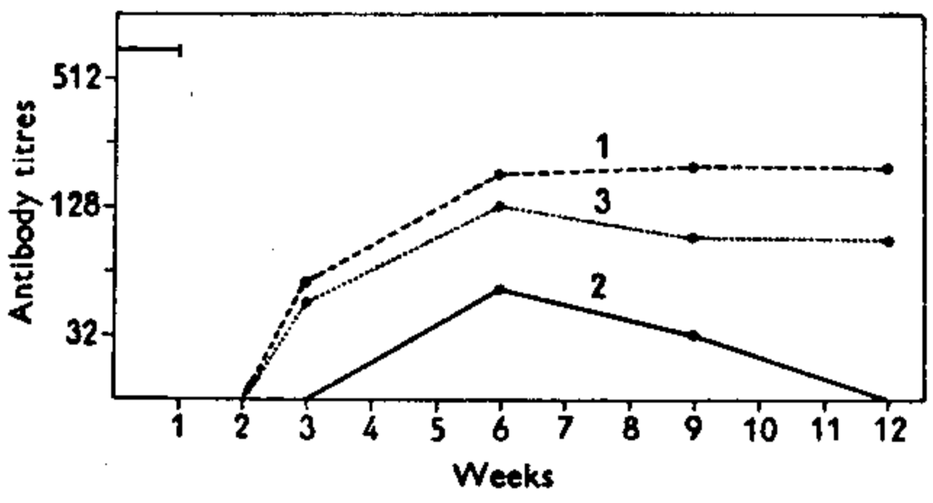

Fig. 7. Geometrical means of HI antibody titres in rabbits immunized with influenza virus emulsified in peanut oil (curve 1). Curve 3 shows the geometrical means of HI antibodies after the administration of procaine penicillin (50,000 units daily for seven days) and curve 2 the values after the administration of tetracycline (100 mg. daily for seven days).

In the infection experiment the effect of tetracycline on the formation of neutralizing antibodies against herpes simplex virus was studied. Rabbits infected subcutaneously with 1 ml. of a 10% mouse brain suspension containing the herpes

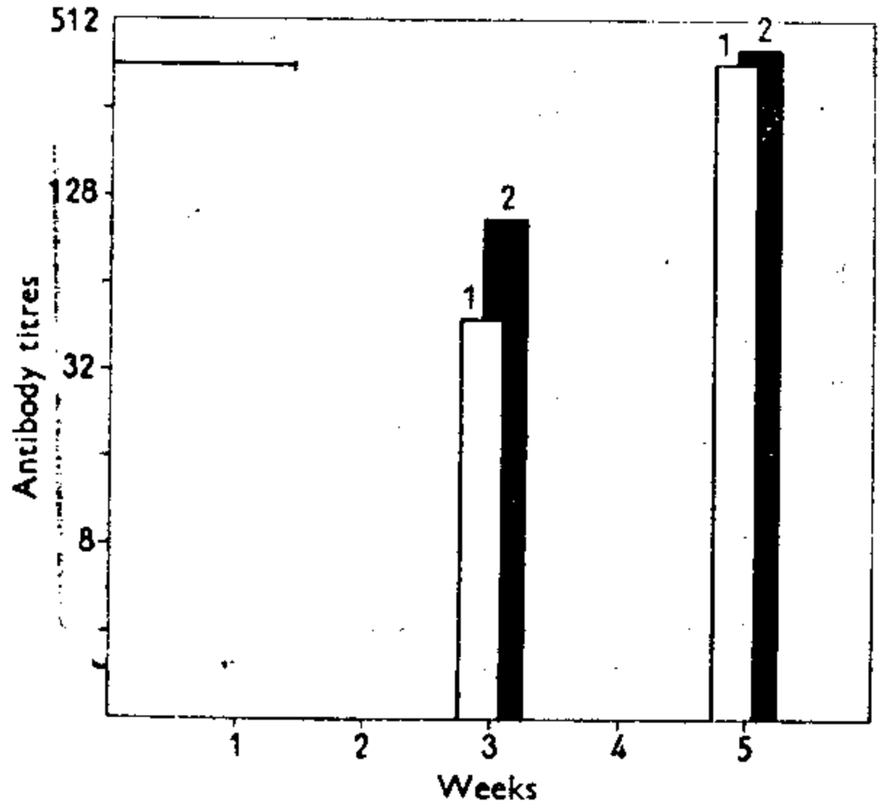

Fig. 8. Geometrical means of complement-fixing antibody titres in guinea pigs immunized with *Coxiella burneti* emulsified in liquid paraffin (2) and in guinea pigs immunized in the same way, to which tetracycline (1) was administered for ten days

strain HC received 25 mg. tetracycline daily (intramuscularly) for ten days from the day of infection.

The neutralization tests with pooled sera collected 28 days after immunization, which were carried out intracerebrally in white mice, showed that the neutralization index of pooled sera from the control rabbits was 1,076, while in sera from rabbits treated with tetracycline it was only 315.

Preliminary experiments showed that in intracorneally infected rabbits with marked keratitis, the administration of tetracycline (25 mg. daily, intramuscularly) is followed by lethal encephalitis within seven days from the onset of keratitis, whereas in control rabbits the interval is ten days.

DISCUSSION

The experimental data show that the depressor effect of antibiotics is directly correlated to the dose of broad-spectrum

antibiotics in particular. The gradated character of the phenomenon shows that it is not merely an experimental coincidence. In Stevens' experiments (1953), in which the intensity of primary response was proportionate to the loss of iodine-labelled bovine gamma globulin, a positive result was obtained by the daily administration of 100 mg. doses of tetracycline antibiotics to the experimental animals. The present authors are aware that this range of over-dosing with tetracycline antibiotics only rarely has a parallel in clinical practice.

Slanetz (1953, 1954), who studied antibody formation following the addition of antibiotics to the animals' food, emphasized the importance of long-term administration of the antibiotic.

Attention was drawn to the importance of the time relationship between the first dose of antibiotic and the first immunization stimulus by Carpenter *et al.* (1955).

It is obvious that antibiotics have different effects on different types of antibodies. Kokushkina and Yegorova (1959) found a discrepancy between circulating antibodies demonstrated serologically *in vitro* and the protective activity of the sera. Marchal, Nicot and Fery (1949) found a difference in the formation of anti-O and anti-H agglutinins after the administration of penicillin and streptomycin.

The present authors showed that the administration of an antibiotic from the moment of immunization can influence the level of antibodies of the agglutinin type, HI antibodies and neutralizing antibodies. The CF antibody level is not greatly affected by the administration of tetracycline.

One hundred-gramme doses of tetracycline depress antibody formation to a greater degree after immunization with influenza virus than after immunization with Brucella antigen. The systematic administration of tetracycline also modifies the primary immune response in the initial phase of long-acting immunization, in which the antigen persists over a long period in an encapsulated granuloma, which (in rabbits) is itself a source of a stable type of antibody.

The findings of Lamensans and Farhi (1955, 1956), who demonstrated that the treatment of unvaccinated infected animals with antibiotics led to their survival, but left them incapable of coping with further infection, are of clinical and practical importance.

The depressor effect of tetracycline in some respects resembles the effect of cortisone on antibody production. Cortisone interferes primarily in the inductive phase of antibody formation, which is very sensitive to substances which damage metabolic functions. From the above experiments it is evident that the administration of tetracycline affects the initial phase of the productive period, i. e. the interval immediately following the inductive phase. Administration of the antibiotic when the productive phase is fully developed is ineffective, as demonstrated by Carpenter *et al.* (1955).

Depression of macrophage activity by antibiotics, discussed by Stevens (1953) also has a parallel in cortisone experiments. It has been demontrated (Fagreus, 1952) however that cortisone activates the phagocytic activity of macrophages, but inhibits the destruction and resorption of phagocytosed antigenic material. With both cortisone and tetracycline, inhibition of antibody formation is only a temporary phenomenon. This is also seen in the experiment in which agglutinin levels reached the same values as in the control animals two weeks after discontinuing both tetracycline and cortisone. It was found that the administration of tetracycline considerably reduced the delayed allergic reaction in sensitized animals. This again is analogous to the disappearance of the delayed allergic reaction always observed after the administration of cortisone.

The authors studied the extent to which antibody formation can be influenced by tetracycline. The question of

why, according to the results of a number of authors, a series of antibiotics with completely different chemical configuration and effect should inhibit antibody production, still remains open.

SUMMARY

The effect of tetracycline on antibody formation was studied in experiments in which different types of antigen, emulsified in lipoid adjuvant, and an inactivated Brucella suspension (*Brucella abortus*) were administered intramuscularly to rabbits.

(1) In rabbits immunized with *Brucella abortus* emulsified in liquid paraffin, antibody formation was depressed by the daily intramuscular administration of 25 mg. tetracycline for eleven days from the day of immunization. Tetracycline also markedly depressed the intensity of the delayed allergic reaction to the intradermal administration of Brucella bacterin.

(2) On increasing the daily dose of tetracycline to 40 mg. (for 14 days), using the same immunization technique, the antibody level curve was the same.

(3) The inhibitory effect of cortisone on antibody formation was more marked than the effect of tetracycline.

(4) Inhibition of the primary immune response in rabbits immunized with corpuscular Brucella antigen, to which 40 mg. tetracycline was administered daily for 14 days, was relatively little marked.

(5) Depression of antibody production was most pronounced after immunization with Brucella and influenza antigen, on increasing the daily dose of tetracycline to 100 mg., administered for 7—12 days.

In an infection experiment the effect of tetracycline on the formation of neutralizing antibodies against herpes simplex virus was studied. In pooled sera from rabbits not treated with tetracycline, the neutralization index was 1,076, while in rabbits to which tetracycline was administered it was only 315.

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ОПЫТЫ ВОЗДЕЙСТВИЯ ТЕТРАЦИКЛИНОМ НА ОБРАЗОВАНИЕ АНТИТЕЛ

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В опытах внутримышечного введения различных типов антигенов, эмульгированных в липоидном adiuvans, и суспензии инактивированных Brucella abortus исследовалось действие тетрациклина на образование антител.

(1) У кроликов, вспомогательно иммунизированных B. abortus, эмульгированной в парафиновом масле, можно добиться угнетения образования антител путем ежедневных внутримышечных впрыскиваний 25 мг тетрациклина (в течение 11 дней со дня иммунизации). Тетрациклин выразительно угнетает и интенсивность поздней аллергической реакции на внутрикожное введение бактерина B. abortus.

(2) Аналогичный характер носят и кривые уровней антител при повышении ежедневной дозы тетрациклина до 40 мг (в течение 14 дней) с сохранением той же схемы иммунизации.

(3) Подавляющее действие кортизона на образование антител более интенсивно, чем действие тетрациклина.

(4) Сравнительно мало выразительное угнетение первичной реакции иммунитета было получено у кроликов, иммунизированных корпускулярным антигеном B. abortus, которым в течение 14 дней ежедневно вводили по 40 мг тетрациклина.

(5) Наиболее выразительное угнетение продукции антител при иммунизации бруцеллезным и гриппозным антигенами было получено при повышении ежедневной дозы тетрациклина до 100 мг (введение в течение 7—12 дней).

В опытах заражения исследовали влияние тетрациклина на образование вируснейтрализующих антител против Herpes simplex. Индекс нейтрализации в смеси сывороток кроликов, не подвергавшихся действию тетрациклина, представлял 1076, тогда как индекс нейтрализации в сыворотках тетрациклинированных кроликов составлял только 315.