4. CONCLUSÕES

Os resultados dos ensaios realizados e o que se sabe acerca da matéria em causa, permitem-me chegar às seguintes conclusões:

1.ª — A acção imunológica exercida pelos anticorpos sobre as bactérias depende da extensão da superfície destas atingida durante a reacção;

a) A que origina *in vivo* a imunidade específica resulta da acção antigénio/anticorpo, qualitativa/quantitativa, numa grande extensão da superfície; é tanto mais acentuada quanto maior for a área atingida, sendo máxima no bloqueio total dos antigénios;
b) A que determina o *immunological enhancement* deriva igualmente da reacção antigénio/anticorpo, qualitativa/quantitativa, mas em menor extensão daquela superfície.

2.ª — A acção imunológica depende da variação antigénica na dinâmica das populações bacterianas, ou seja, do ambiente em que se processa o crescimento.

3.ª — A acção imunológica antibacteriana determina, dada a alteração acentuada da parede celular e, portanto, da permeabilidade, *cresci-*

mento desequilibrado, consequentemente mais lento; nas estirpes que dividem apenas num plano, traduz-se, *in vitro,* pelo maior volume do elemento bacteriano e pela formação de cadeia, ou aumento da extensão desta; naquelas que o fazem em mais de um, traduz-se pelo aumento da extensão dos agregados celulares. A acção imunológica condiciona, assim, *in vivo,* a imunidade específica, uma vez que a bactéria, perdendo a sua capacidade invasora, se comporta como avirulenta, sendo então inibida pelas defesas normais do organismo. Acresce ainda que a bactéria sensibilizada pelos anticorpos se torna mais susceptível aos mecanismos de defesa.

4.ª — A cadeia e o aumento de volume dos elementos bacterianos são consequência de *crescimento desequilibrado* devido a condições desfavoráveis decorrentes da acção imunológica dos anticorpos.

5.ª — A acção imunológica — *immunological enhancement* — resulta de leve alteração da parede celular pelos anticorpos, tornando mais fáceis as trocas nutritivas e originando assim um mais rápido crescimento, quer *in vivo,* quer *in vitro,* principalmente quando este se processa em condições desfavoráveis.

6.ª — Dado o conhecimento da acção imunológica antibacteriana é agora possível proceder a estudos *in vitro,* até aqui não realizáveis ou apenas exequíveis *in vivo,* a saber:

a) Identificação imunológica de estirpes bacterianas, incluindo as avirulentas;
b) Apreciação do grau de imunidade das populações;
c) Verificação do poder imunizante dos soros específicos;
d) Avaliação do grau de imunidade conferida por vacinas e, implìcitamente, do valor destas;
e) Instituição de esquemas mais válidos de imunoprofilaxia e imunoterapêutica.

A identificação imunológica das estirpes deverá ser controlada por estirpe devidamente estudada; a apreciação do valor do soro imune sê-lo-á por soro padrão ou de reconhecido valor. Além disso, deverão ainda ser estudadas em relação a cada caso, e nas condições de ensaio, as variações da D. M. I., significativas de diferença imunológica.

7.ª — Na identificação imunológica das estirpes de crescimento rápido, a variação da D. M. I. 2 × parece ser representativa de diferença de tipo imunológico; nas estirpes cujo crescimento se processa normalmente em cadeia, mais lentamente, a variação da D. M. I., dentro do mesmo tipo imunológico, é mais acentuada, principalmente em relação às estirpes atenuadas.

8.ª — De maneira geral, os soros que *in vivo* protegem consoante os respectivos métodos de apreciação exercem *in vitro* acção imunológica antibacteriana, na fase logarítmica, na diluição 10^{-2}.

9.ª — Comummente, os animais vacinados, cujos soros exercem acção imunológica antibacteriana *in vitro,* na fase logarítmica, na diluição 1/3, encontram-se protegidos nas condições de apreciação *in vivo* das respectivas vacinas.

10.ª — Dada a variação antigénica nas populações bacterianas em relação ao ambiente de crescimento, torna-se necessário realizar os ensaios imunológicos *in vitro* — principalmente os que respeitam ao homem e animais — em meios de cultura adicionados de soro normal de animal da espécie em causa, em concentração superior a 1/10, a fim de aproximar tanto quanto possível os ambientes de crescimento, *in vivo* e *in vitro,* e, consequentemente, tornar mais semelhantes, antigènicamente, as populações, com vista à obtenção de resultados mais válidos em relação aos *in vivo.*

11.ª — O organismo animal representa, para os microrganismos, ambiente complexo, dada a sua estrutura multicelular e a diversidade de órgãos e tecidos que o compõem, de onde a variação antigénica na dinâmica da infecção, podendo esta ser detida sob a acção imunológica dos anticorpos em qualquer estádio da sua evolução. A infecção/imunidade específica é, por conseguinte, de natureza dinâmica.

12.ª — No que concerne os agentes bacterianos determinantes de infecções em que a imunidade celular representa o principal factor da resistência específica, os anticorpos exercem, da mesma forma, acção imunológica — crescimento em cadeia. As infecções desta natureza, em geral *menos agudas, resultam, portanto, da capacidade de vida intracelular dos respectivos agentes infecciosos; daí, ser fundamental à resistência específica* a imunidade celular consequentemente adquirida.

13.ª — Em geral, verifica-se certo grau de relação entre susceptibilidade e resistência natural à infecção por determinado agente bacteriano e sua capacidade de crescimento *in vivo* em meio de cultura com soro normal de animal da espécie em causa, em concentração superior a 1/10.

14.ª — O que se passa com as bactérias, sob o ponto de vista imunológico, ocorrerá igualmente com os vírus, parasitas e células, apenas com as diferenças inerentes à diversidade dos respectivos mecanismos vitais.

SUMMARY

This work is based on the antibacterial immunological action exerted, *in vitro,* by specific sera. This action shows itself through the growth in chains of strains that normally divide in one plan or the increase of the bacterial packets of the strains normally divide in more than one plan. As far as the strains normally dividing in chains, *in virto,* are concerned, this action is shown by an increase in the cells' numbers.

Specific sera without immunising power agglutinate only the bacterial cultures.

Based on this knowledge, various aspects of immunity related with antibodies might be faced and studied *in vitro,* as for instance:

a) Immunological identification of bacterial strains;
b) Immunological power of specific sera;
c) Immunising power vaccines by the immunological action of the sera of vaccinated animals;
d) Immune state of the populations, etc.

The technics followed consisted in promoting the growth of the bacterial agent in the presence of specific type serum (without preservatives). Each test is duly controlled by the same strain cultured in the presence of normal serum and in the same conditions of the experiment.

From the work carried out the following conclusions can be taken:

— The culture medium and the incubation temperature are variable, depending on the bacteria and must correspond to the best growth conditions *in vitro*. On being plated, the media must be at incubation temperature.

— The incubation period must normally allow the stationary phase of the growth cycle to be reached. It is therefore also a variable to be considered, depending of the bacteria species and strain.

— The volume of the medium might vary according to the work carried out and the type of test.

— The amount of the culture to be plated must not change; 0,01 c. c. per c. c. of the medium (10^{-2}) is the best for this purpose. The plating from a culture in full logarithmic phase led to a quicker test.

— The concentration of the specific serum in the test must change, and the culture, as has been said, must be fixed. The simultaneous variation of both has been studied, but we could not get more significant and valuable results. The increase or decrease, within limits, of the cultures' or sera' amounts influences the dynamics of immunological action only in connection with time.

In the case of hyperimmune sera, the highest concentration will be 0,1 c. c. per c. c. of medium (10^{-1}); nevertheless, it will become smaller according to the immunising power of the serum. Usually, in the tests carried out, the concentration of the serum varies in base 10 — from 10^{-1} to 10^{-5}. In tests with immune sera — sera of vaccinated animals — the concentration, in the culture medium, varies, generally, from 1/3 to 1/9.

— Microscopic observation of cultures: — It consist of a direct examination with an optical microscope (600 ×) of slide preparations of the culture after staining with toluidine blue. The drying of the preparations should be carried out in the incubator at 37°C and the fixation is performed with ethyl alcohol. The examination of cultures submited to the immunological action of specific sera should be done in the growing period, preferably in the logarithmic phase, as later on there will be a strong agglutination which, in a way, will make difficult the observation of the chain. The right time for the examination depends of the bacterial species

but, with the strains used in the tests, with the exception of *Brucella,* the observations were normally carried out between 2 and 10 hours after plating and after 16 or 24 hours. When a study of immunological action during the whole growth cycle of a bacterium is wished, the observations' intervals should vary according to the species.

— Minimal immunological dose (M. I. D.): — The smallest dose of specific serum which produces antibacterial immunological action (either growth in chains or a significant lenghtning of chains in strains that grow in this way). It is ascertained during the logarithmic phase, full growth, or the last phase of growth. It is reckoned that the chain has a significant lenghtning when it reaches an average of, at least, 2 times its lenght in normal serum.

— Finding the immunising power of specific serum, *in vitro*: — This should of recognised value *in vivo* which serves as a standard. The M. I. D. should be found in the logarithmic phase, in the full growth period and the conditions above mentioned. If a more complete knowledge of the serum's value should he contemplated, then the apreciation should be carried out in function of M. I. D., both in the logarithmic and the last phase of growth. It should also be performed with media with a high concentration of normal serum from an animal of the same species of the *in vivo* test, so as to get together, as far as possible, *in vitro* and *in vivo* conditions and, therefore, obtain less divergent bacterial populations in order to arrive at more significant and valid results.

— Immunological *in vitro* identification of bacterial strains: — The immunological identification of bacterial strains takes for granted, in connection with specific serum under which action they grow *in vitro,* an identical behaviour to the type strain, under the same conditions. Variations of M. I. D. within the same immunological type depend on the bacterial species and strain, as is conveyed by the description of the test. In the strains which, as isolated elements, grow rapidly, reaching their full growth after 4 a 6 hours, the 2 × M. I. D. variation seems to point to a difference of immunological type. In strains where the growth is slower or done in chains, the variations, within the same type are more clear and dependent of the strains itself.

Concerning bacterial species which strains form only one immunological type (*B. anthracis, E. rhusiopathiae,* etc.) the *in vitro* immunological identification corresponds to the bacteriological diagnosis.

Sera to be used in the immunological identification of bacterial strains and in testing the immunising power of specific serum for prophylaxis and therapeutics should have, at least, 100 M. I. D. per c. c., according to tests performed during the logarithmic phase — full development.

The technics of the *in vitro* antibacterial immunological action must be submited to *in loco* adaptation tests, as is only natural, and its results will be more significant when analysed under statistical methods, nowadays universally followed.

On its side, the microtechnic in cinematography will show, in the whole growth cycle, the dynamics of immunological action.

The several serological manifestations resulting from the *in vitro* antigen-antibody reactions (agglutination, complement-fixation, precipitin test, etc.) are not representative of the immunological antibacterial action as they not connected with immunity.

With this simple, economic and quick technics, it is obtained what has not been possible *in vitro,* until now: the close relationship with the results of specific immunity. With it, it is now possible the immunological identification of strains avirulent to test animals and also the testing of sera and vaccines used mainly for human prophylaxis and that, on account of the challenge strains being avirulent, cannot be tested *in vivo.* On the other hand, testing in man himself and in the laboratory, besides being difficult, do not always lead to significant results chiefly with whooping cough, typhoid and paratyphoid, cholera and bacillary dysentery. It should also be pointed out that it is now possible to study the immunising power of mixed vaccines in the same animals' group thanks to the testing of *in vitro* antibacterial immunological action of the vaccinated animal's serum.

The work carried out and described in this paper is a contribution to the knowledge of anti-infectious and anti-cellular specific immunity. The immunological action comes as a result of the antigen-antibody, qualitative-quantitative reaction in part of the bacterial surface and depends of antigenic variations in he populations dynamics in the environment were the growing takes place.

The specific infection/immunity is thus a process of a dynamic nature. This leads now to the solutions of a number of problems of prophylaxis against infectious diseases by active and passive immunisation, and ought to be accounted for in the immunopropylaxis of malignant change.

Within the same theory it is now possible to explain the *immunological enhancement* found mainly in the malignant change in which the antibodies paradoxically help the cellular growth instead of stoping it. It is probably a qualitative-quantitative aspect of the antigen-anlibody reaction in the cellular surface, but put forward in a lesser extent conducting it to immunity; nevertheless both of them depend on the same antibodies and, consequentely, on the same antigenes.

CONCLUSIONS

1st — The immunological action of antibodies on the bacteria depends on the greater or lesser surface reached in the reaction.

a) That due to the specific immunity results from the antigen--antibody, qualitative-quantitative reaction on a larger part of the bacterial surface; it is the more intense, the greater the area reached; and it is the greatest in the complete blocking of the antigenes.

b) That due to the *immunological enhancement* derives also from the antigen-antibody, qualitative-quantitative reaction, but in a smaller extent of that surface.

2nd — The immunological action depends on the antigenic variation in the bacterial population's dynamics, that is in the environment on which the growth takes place.

3rd — The antibacterial immunological action causes a slower *unbalanced growth* due to the marked alteration of the cell wall and its permeability; in the strains dividing in one plan only, it shows itself, *in vitro,* by the biger volume of the bacterial element and by the formation of the chains of their lenght's increase; in those dividing in more than one plan, it takes the form of biger cell aggregates. Thus, the

immunological action conditions *in vivo,* the specific immunity, as the bacterium, once last its invading power, behaves as avirulent, being then inhibited by the normal body defences. It should also be born in mind that the bacterium sensitized by the antibodies is more sensitive to the defence mechanism.

4th — The chain and the increase in volume of the bacterial elements are a result of the *unbalanced growth* due to the unfavourable conditions resulting from the immunological action of the antibodies.

5th — The immunological action — *immunological enhancement* — results from a slight alteration of the cell wall by the antibodies, which makes it easier the nutritive changes and so a quicker growth both *in vivo* and *in vitro,* mainly when it takes place in unfavourable conditions.

6th — Due to our knowledge of the antibacterial immunological action, it is now possible to carry out work *in vitro* which, up till now, has not been possible or only so *in vivo,* as for instance:

a) Immunological identification of bacterial strains, including avirulent ones;

b) Estimation of the immunity's degree of the populations;

c) Estimation of the immunising power of specific sera;

d) Estimation of the immunity's degree confered by vaccines and, implicitly of their value;

e) Building up of more valid immunoprophylatic and immunotherapeutic schemes.

The immunological identifications of strains must be controlled by a well known strain; the estimation of the immune serum's value must be controlled by a standard serum or one of recognised value. Besides that, the variations of M. I. D. resulting from immunological distinction, should be studied for each case and in the conditions of the test.

7th — In the immunological identification of quick growing strains, the variation of M. I. D. 2 × seems to be representative of an immunological type difference; in the strains normally growing more slowly, in chains, the M. I. D. variation, within the same immunological type, is more pronounced, mainly in relation to attenuated strains.

8th — Generally speaking, the sera which, *in vivo,* protect according to the methods of evaluation, produce, *in vitro,* an antibacterial immunological acion, in the logarithmic phase, in a 10^{-2} dilution.

9th — Vacinnated animals with sera producing an antibacterial immunological action *in vitro* in the logarithmic phase, in a 1/3 dilution, are usually protected in the *in vivo* evaluation conditions of their vaccines.

10th — Due to the antigenic variation in the bacterial populations, in relations to the growth environment, it is necessary to carry the immunological tests *in vitro* — mainly those of man and animals — in culture media added with normal serum from an animal of the same species, in concentration higher than 1/10, in order to get, as near as possible the growth environment both *in vivo* and *in vitro,* and consequently, making antigenically more similar the populations, in order to obtain more valid results, in relation to those *in vivo.*

11th — The animal body presents, to microorganisms, a complex environment, due to its multicellular structure and diversity of organs and tissues; all this has, as a result, an antigenic variation in the infection dynamics, which may be stoped under the immunological action of the antibodies in every stage of its evolution. The infection/specific immunity is, therefore, of a dynamic nature.

12th — Concerning the bacterial agents of infections in which cell immunity represents the main factor of specific resistance, the antibodies give rise, in the same way, to an immunological action — growth in chains. The infections of this type, generally less acute, result, therefore, from the intracellular living capacity of their own infectious agents. Thus, it is fundamental to the specific resistance the cell immunity so acquired.

13th — In general, it is found a certain relationship between the susceptibility and natural resistance to infection by a bacterial agent and its growth capacity *in vitro* in culture medium with normal serum of the species of the animal under study, in concentrations higher than 1/10.

14th — What happens with bacteria, under the immunological point of view, takes also place with virus, parasites and cells only with differences due to the diversity of their vital mechanisms.

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