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POISONING

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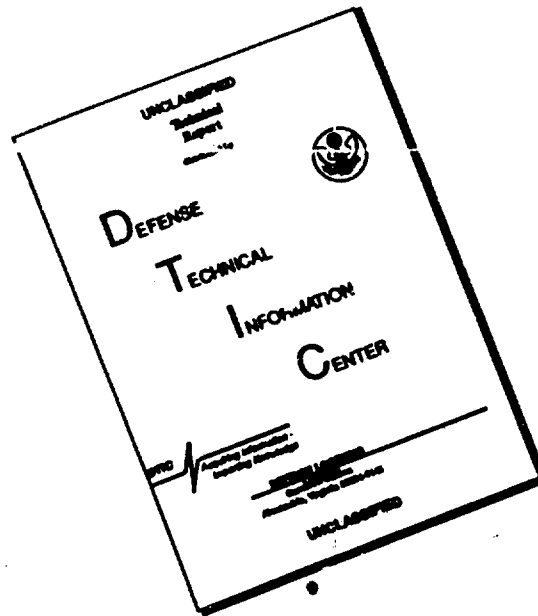
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A VACCINE AGAINST ORGANOPHOSPHORUS POISONING

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Effectiveness of a vaccine against organophosphorus poison-
ing requires that it evoke quantities of antibodies specific for the
agent. Importantly the average specific binding affinity per antibody
molecule must be high to insure that the poisonous agent binds with
the antibody more rapidly than with the normal target of poisoning,
namely cholinesterase.

A foreign molecule, when injected, will be recognized selec-
tively by a few lymphoid cells possessing on their surface specific
antigen recognizing units. Some of these specific lymphoid cells in
further processes elaborate information that will tell other cells
whether to produce specific antibodies or suppress the formation of
specific antibody. If the former occurs we say our injected molecule
is immunogenic; if the latter occurs we say it is tolerogenic. To
produce a good vaccine against organophosphorus poisoning we had to
modify the organophosphonate molecule to make it more immunogenic than
tolerogenic. We used fundamental information initiated by other im-
munologists many years ago and perfected more recently in several lab-
oratories (1). Two things are necessary. The first is to increase
the molecular size of the specific antigen, organophosphorus. This is
accomplished by coupling it covalently to a large molecule such as a
protein which we will call "carrier." The second requirement is the
use of a protein carrier which in itself embodies antigenicity in
excess of tolerogenicity. After many years of trial we have now
selected the protein carrier as well as the system for conjugation
of agent to carrier which yields large amounts of antibodies with
high binding affinity in every animal injected and which consequently
protects every animal against exposure to the agent. We will discuss
only this system.

The agent is the organophosphonate E600 (paranitrophenyl-
diethylphosphate). Its formula is in the upper right hand corner of
figure 1. To make an antigen the agent is coupled to a protein. To
this end the nitro group is reduced to the amine resulting in a non-

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toxic compound. Among the various choices for coupling with amine to
protein the only one that gave a regularly good antigen was diazoti-
zation followed by coupling into protein tyrosine at ratios of 3 to
15 molecules of agent per 100,000 molecular weight units of protein.
The only regularly effective protein carriers have been purified
lobster hemocyanine and aggregated bovine gamma globulin, each having
a molecular weight in excess of 4 million.

The antigen or vaccine so produced (table I) was given to rabbits
in 3 or 4 intravenous injections. This was not the optimal way of
animal immunization but we have chosen it as the only one practical
in a human population. Despite this, using the properly prepared
antigen, we obtained a specifically precipitating antibody response
in excess of 100 µg nitrogen per ml in each rabbit injected. Further-
more each rabbit possessing this antibody was fully protected against
2 to 4 intramuscular LD50's of the agent E600. By full protection we
mean that the animal was entirely asymptomatic after exposure to agent.
Control rabbits of course had convulsions in each case and were dead
within the hour.

Thirty-one weeks after the last injection of antigen the level
of circulating antibody had waned to below detectability. Neverthe-
less, upon exposure to 4 LD50's of agent, such rabbits lived longer
than unvaccinated controls.

Immunologic prophylaxis differs from that obtained with oxime
and atropine in several respects. Oxime-atropine prophylaxis, while
sometimes preventing death, will not prevent the patient's symptoms.
He will be very sick indeed, hardly able to cling to life. Immuno-
logic prophylaxis prevents all symptoms from agent. There is reason
to believe that an exposed patient would never know that he had been
exposed.

Oxime-atropine prophylaxis is only effective if high blood levels
of these compounds are obtained. Because they are eliminated by the
body, these drugs must be given at a predictable instant prior to an
enemy attack. Immunization on the other hand protects for a long
time and therefore prediction of the time an enemy attack will occur
is not necessary.

The data show that immunization is a predictable, effective and
simple prophylaxis so long as antibody production is successful.
According to our experience it is successful once we find the best
mode of conjugation and the best carrier. We have not yet found
these in the case of agent GD. The right lower part of figure 1
gives the formula of GD. For immunization the nitrophenyl analog of
GD was produced bearing a nitrophenyl group instead of the fluorine.
The compound was reduced and coupled into carrier protein tyrosine
via diazotization as shown in figure 1, or conjugated with protein
lysine with carbodiimide. So far immunized animals have not been
protected against GD. This failure has nothing to do with the

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TABLE I

Group (3 rabbits/group)	Antigen 800 moles agent derivative/mole hemocyanin 10-30 mg/kg iv	Exposure to E600 (subcutaneous)	Average time to death after exposure
E_1	3 x at 25-30 day intervals	2 x LD50 (1 week after 3rd antigen)	No ill effect
Control	-	2 x LD50	14 min
E_1	4 x at 25-30 day intervals	3 x LD50 (1 week after 4th antigen)	No ill effect
Control	-	3 x LD50	55 min
E_1	4 x at 25-30 day intervals	4 x LD50 (31 weeks after 4th antigen)	107 min
Control	-	4 x LD50	45 min
E_2	4 x at 21 day intervals	4 x LD50 (1-3 weeks after 4th antigen)	No ill effect
Control	-	4 x LD50	73 min

Protected animals not only survived but, in contrast to other modes of prophylaxis, had no ill effect from the agent.

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toxicology of GD but has been clearly correlated with a low anti-
genicity of the vaccines we have produced until now, resulting in
only minimal levels of circulating antibodies.

If a human population is immunized one cannot monitor the ef-
fectiveness of immunization by resistance to agent exposure because
of the danger involved. Instead one has to measure two parameters.
One is the level of circulating antibody in terms of antibody nitro-
gen. The other is the average intrinsic association constant of
these antibodies, which is a measure of their binding strength and
clinical effectiveness. The amount of antibody is being monitored
by the use of agent coupled to bovine serum albumin. This antigen
is reacted with antibody, the resulting complex removed by ammonium
sulfate precipitation and the residual supernatant antigen assayed
spectrophotometrically. For measuring the average binding strength
of antibody, in one approach, purified antibody is needed. Anti-
E600 has been purified by removing antibody from serum with agent
covalently bound to a derivatized dextran column and eluting the
antibody with an excess of agent. Agent is then removed from puri-
fied antibody by exhaustive dialysis. When varying amounts of agent
are added to known amounts of purified antibody the ratio of free
to bound antibodies depends on the average intrinsic association con-
stant. The amount of bound agent was measured by quenching of the
fluorescence of the purified antibody. Free antibody absorbs light
maximally at 280 nanometers and emits the energy at about 350 nano-
meters. Agent E600 absorbs at 350 nanometers and hence, when bound
in the antibody molecule, quenches its fluorescence emission. The
average intrinsic association constant of purified anti-E600 was
found to be 5×10^5 and its heterogeneity index 1.0. This indicated
surprisingly weak binding and superior homogeneity. We concluded
that dialysis failed to remove all the agent from antibody during
purification and that purification in itself selected antibodies of
low binding strength.

To obviate the need for purified antibody and the incumbent
selection of binding properties, we prepared agent coupled to tyrosine
and reacted the product with ^{125}I. Equilibrium dialysis against an
IgG fraction of antiserum permitted evaluating bound radioactivity
counts in a system that does not require purified antibody. The
average intrinsic association constant was found to be higher than
10^{10} and the heterogeneity index about 0.6, comparable to the highest
binding strength systems known to immunology. The protective effect
of the antibodies produced by immunization is thereby explained.

The antibodies and the immunity evoked are specific to the agents
used. There is little cross-reactivity with other organophosphates.
This of course is the expected result and is a corollary of high bind-
ing activity and clinical effectiveness. Immunization has thus become
an effective new principle for prophylaxis against organophosphonate
poisoning. The procedure is independent of oxime resistance of the
agents and in any event it is more effective than previous attempts

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at prophylaxis against organophosphonate poisoning. Therefore, the major problem at this time is not effectiveness, but safety. Although no rabbit has died as a result of immunization, we must realize that clinical vaccination is not given to a few rabbits but to thousands of people. It is imperative that none of them has acute or chronic side effects from the vaccine.

Our present vaccines not only evoke antibodies to the agent, but even more so to the carrier protein. Repeat injections of vaccine will form carrier antibody complexes capable of causing immune complex nephritis, a chronic and fatal disease in man. Even though in animals under experimental conditions only transient immune complex nephritis has been produced, followed by complete recovery upon cessation of injection of antigen, we cannot assume that in a patient with subclinical lupus erythematosus or subclinical and hence unrecognized nephritis, immunization will not precipitate a progressive lethal syndrome. The proper avoidance of this potential, delayed complication is the design of vaccines that produce high agent and low carrier immunity. This requires the simultaneous and independent variation of two factors, each difficult to optimize, and hence not measurable by the single analysis of success of random immunization in a limited number of animals. Instead, a test to predict the response with different agent carrier combinations must be developed. Recent work by Benaceraff's group (3,4) and by others (5) has shown that in the ligand carrier response a different cell may recognize the carrier and the ligand specificities and that both cells cooperate towards the function of a mature antibody-producing cell. To study this cooperation on a large scale we cannot wait until cells actually have produced measurable amounts of antibody. Instead we should be able to trace the process in an earlier stage by checkerboard titration and to predict the kinds of antibodies to be produced as a result of in vitro admixtures of cells recognizing varying carriers and varying agent derivatives. To this end we must be able to map antigen receptors on the surface of the immunocompetent cell. We must also be able to map nascent antibody and its specificity on the surface and in the interior of the cell slated to become an antibody-producing cell. Essentially, this is a requirement for an immuno-histochemical method which is sensitive and specific enough for recognition of antigenic determinants within a single molecule and for quantitatively enumerating single molecules. Methods that use antibody conjugated with a detector, such as fluorescein or ferritin, did not possess the requirement (6). Hence, we introduced a new principle for immunohistochemistry in which only native, unlabeled antibodies are employed and in which the antigen antibody reaction on electron microscopic tissue sites is amplified by an enzymatic detector (7-15).

In figure 2 we have depicted the antibody molecule in its familiar "Y" shape in which the fragments bearing the specific combining sites are drawn in bold lines. There are two such fragments in each molecule; hence, two specific combining sites. Tissue antigen

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is first reacted with specific antiserum produced in a suitable
species such as a rabbit. Since diluted antisera usually are used
the antibody combines with the tissue antigen via both of its com-
bining sites. In the second step sheep antiserum to rabbit antibody
is applied in excess. The rabbit antibody is now the antigen, and
since the sheep antibody is used in excess, only one of its combining
sites reacts with the rabbit antibody and the other combining site
remains free. Next, purified soluble antigen-antibody complex is
applied, consisting of peroxidase and rabbit antibody to peroxidase.
This rabbit antibody is, however, also an antigen to the sheep anti-
body already attached to the tissue and hence the complex will bind
specifically. The enzyme is then localized electron microscopically
by its substrates, hydrogen peroxide and diaminobenzidine, followed
by osmication of the polymeric enzyme product. By this mechanism
an enzymatic reaction for peroxidase affords amplification to the
detection of the single rabbit antibody molecule initially attached
to the tissue.

We also introduced a procedure for making purified soluble
peroxidase-antiperoxidase complex (8) (figure 3). It depends on
precipitating antiperoxidase from antiserum with peroxidase, re-
dissolving the precipitate at acid pH in a moderate excess of peroxi-
dase, and separating complex from free peroxidase by ammonium sulfate
precipitation. It turned out that the complex consisted of three
molecules of peroxidase and two of antiperoxidase and that it was
pentagonally ring shaped. Extensive physical chemical studies on
the complex have shown it to have the dimensions indicated in figure
3 and have also explained the stability of the 3:2 circular bonds
which exceed those of antigen-antibody complexes of other specifici-
ties.

The next few figures demonstrate that this method indeed reveals
single antigenic sites, thus permitting quantitation (16).

If upon progressive dilution of reagents in an immunohistochemi-
cal procedure the intensity of visualization of a site becomes pro-
gressively fainter, the method is not very sensitive. If the method
is sensitive it is the number of reacting sites that decreases while
the intensity per localized spot remains unaltered. This is exactly
what happened with the n method, now called the unlabeled-antibody
enzyme method. Thus, when antiserum is progressively diluted the
probability increases that each discrete spot represents the amplified
deposit corresponding to a single molecular site in situ.

Figure 4 shows what happens when antiserum to sheep red cells
is applied to the cell followed by the unlabeled antibody enzyme
method. The concentration of the antiserum was 1/50th of that re-
quired for saturation of the cell surface as demonstrated with radio-
labeled antibody and by enzymatic assay. Nevertheless the total cell
surface was specifically localized as a continuous stain.

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When serum is progressively diluted localization becomes discontinuous only after dilutions in excess of 1/5,000th the saturation concentration. For the preparation shown in figure 5, the concentration was 1/500,000th of the saturation concentration. Localization of a single molecule per cell section is evident. In fact there was only one molecule per two sections, or 69 molecules per cell.

By this method we can thus estimate relative numbers of single molecules on different subcellular sites. In order to express results in absolute numbers it is necessary to correlate the data at low concentration which yields discrete localization with data at saturation concentrations. To this end a novel nephelometric method for measurement of peroxidase activity was developed (12). The method is so sensitive that we can estimate specific enzyme activity per localized molecule at the <u>low</u> level of antibodies where single molecules can be discerned in the electron microscope. This is beyond the sensitivity of measurement with radioisotope-labeled antibody. At the same time specific enzyme activity can be measured at <u>higher</u> levels of antibody at which antibody also can be measured by radioactivity. This level is too high for counting single molecules by electron microscopy. Correlation of both specific activities permits expression of electron microscopic data in terms of absolute numbers of molecules.

Our own application of the unlabeled antibody enzyme method concerns the separate detection and enumeration of organophosphonate and of protein carrier molecules in bone marrow-derived, thymus-derived and other antigen-recognizing lymphoid cells. Lymphocytes are being separated for this purpose in zonal gradients and the specific receptor-bearing cells within the fractions identified histochemically. Cells from receptor bearing fractions are then mixed and early formation of antibodies in culture is again monitored histochemically.

Meanwhile our unlabeled antibody enzyme method has found a range of applications in other laboratories. These include cancer research, virology, blood coagulation research, embryologic differentiation, transplantation, hormone secretion, hormone effects, and plant physiology.

Immunization against nerve agents constitutes the first instance that vaccination against a synthetic chemical, not occurring in nature, has been found to be pharmacologically effective. It is hoped that immunohistochemically monitored refinements will make the procedure clinically safe.

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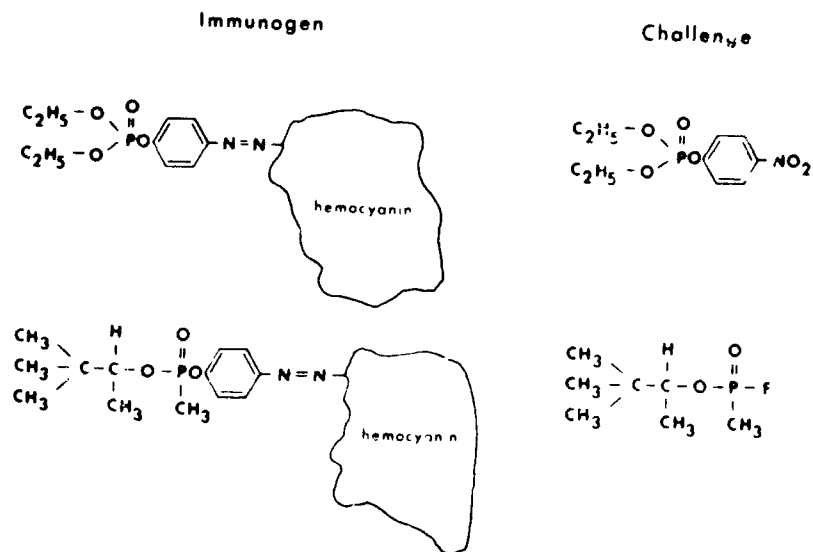STERNBERGER, SIM, KAVANAGH, CUCULIS,
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Immunogen   Challenge

Figure 1. Diazotized Agent (Analog)-Protein Conjugates
           Compared to the Agent.

           (Upper)   Azo-P-Aminophenyldiethylphosphate-
                     Hemocyanin Compared to Paraoxon

           (Lower)   Azo-P-Aminophenylmethylpinacolyl-
                     phosphonate-Hemocyanin Compared to GD

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step 1   rabbit anti-X

step 2   excess sheep antirabbit IgG

step 3   PO - rabbit anti-PO complex
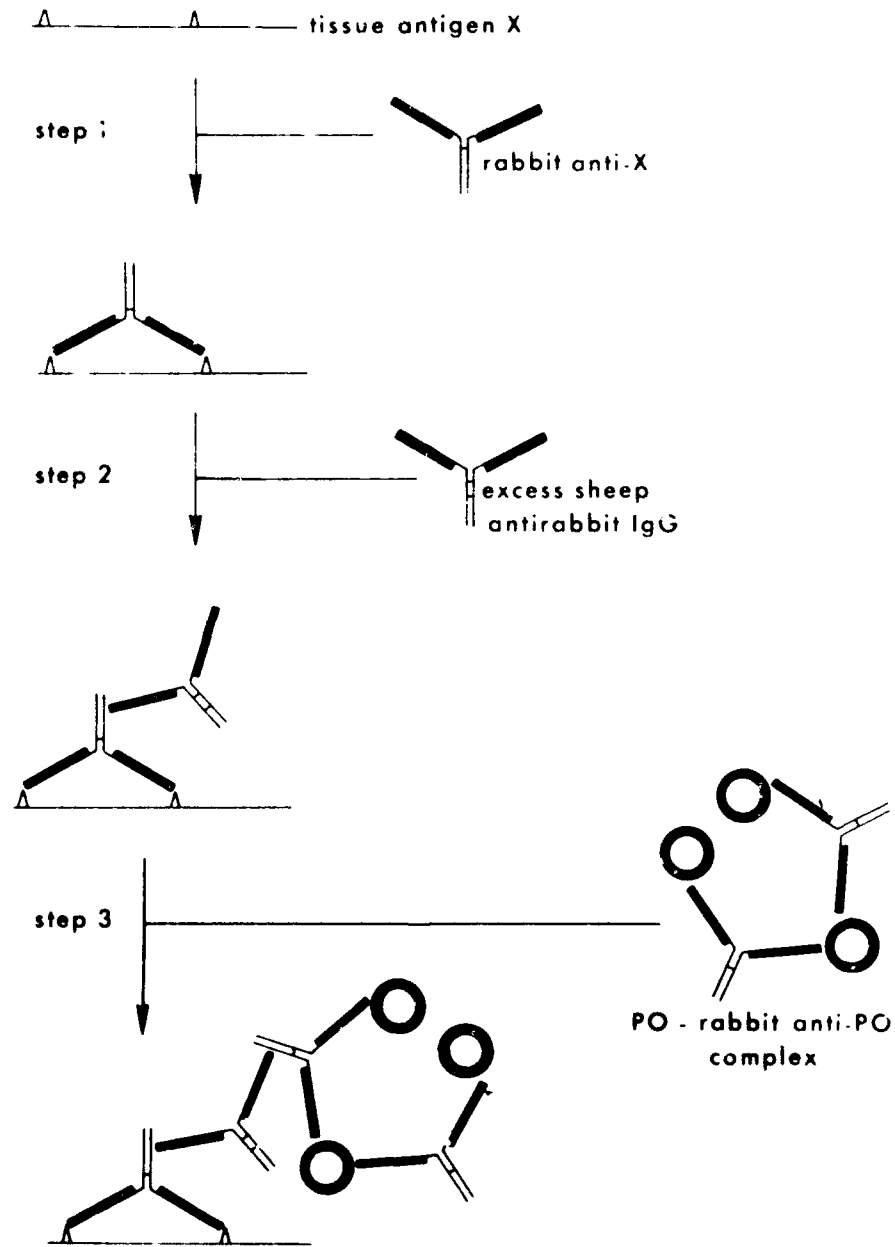
tissue antigen X

Figure 2.   Sequential Application of Reagents in the Unlabeled
Antibody-enzyme Method of Immunohistochemistry.

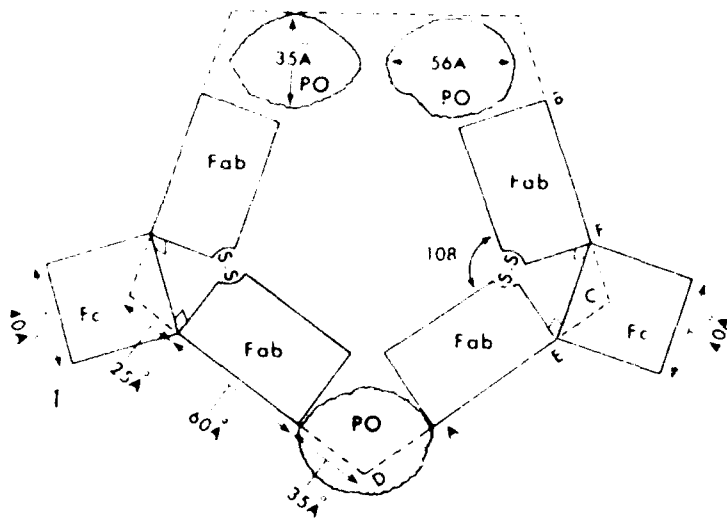439

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Figure 2. Peroxidase-(Rabbit) Antiperoxidase Complex (PAP).

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Figure 4. Electron Micrograph of a Sectioned Sheep Erythrocyte Stained by the Unlabeled Antibody-Enzyme Method Using a 1:50 Dilution of Antiserum.

Figure 5. Electron Micrograph of a Sectioned Sheep Erythrocyte Stained by the Unlabeled Antibody Method Using a High Dilution (1:500,000) of Antiserum.