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## Influence of Antigens on Release of Free Fatty Acids from Arlacel A (Mannide Monooleate). (31435)

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A high incidence of sterile abscess formation in women vaccinated with mineral oil and 7-n-hexyloctadecane emulsified tetanus toxoids from one of two series of preparations was reported by MacLennan *et al*(1). Abscess formation following use of emulsified cholera vaccine(2) and emulsified typhoid vaccine(3) also has been reported. We undertook the present studies to find the cause of these reactions.

Mannide monooleate (Arlacel A, Atlas Chemical Industries) has been used extensively as an emulsifier in the preparation of vaccines and repository allergens for experimental use in man(1-8). Sterile abscess formation observed in recipients of water-in-oil emulsions of influenza virus vaccine(4,9-11) was attributed to the use of impure batches of Arlacel A(4) which had a high content of free oleic acid(11). Subsequently, the lots of Arlacel A to be incorporated in vaccines were tested for freedom from toxicity by the Berlin criteria(12). Nevertheless, adverse conditions of storage and transportation might cause hydrolysis of the ester linkage of the mannide monooleate(12,13) with release of oleic acid prior to the use of the emulsifier. In addition, substances in the antigen might cause the release of free fatty acids in the final biologic product.

The present report shows that the emulsified toxoids and the cholera and the typhoid vaccines which caused sterile abscesses contained free fatty acid (FFA) and that a variety of antigens is capable of releasing free fatty acid from a water emulsion of Arlacel A. This report also contains preliminary data on the characterization of an active substance in *Clostridium tetani* culture filtrate which hydrolyzed Arlacel A.

*Materials and methods. Antigens.* Tetanus toxoids $B_1$, $C_1$, $D_1$, $A_2$, $B_2$, $C_2$ and $D_2$ were kindly prepared by Parke, Davis & Co. for use in the field study in New Guinea(1). The toxoids with subscript 1 were prepared from a common lot of toxoid containing 612 Lf/mg total nitrogen (TN) while toxoids with subscript 2 were prepared from another lot of

toxoid containing 278 Lf/mg TN. For clinical use toxoids $A_2$ and $B_2$ were filled in disposable glass syringes and designated $A_2S$ and $B_2S$. The syringe-filled toxoids had been shipped packed in water ice to and from New Guinea. The other toxoids filled in glass bottles had been stored at 5°C since receipt from the manufacturer in 1962 and early 1964. Four additional emulsified preparations in glass syringes (Parke, Davis & Co.), W, X, Y and Z contained, respectively, toxoids with specific activities of 1370 Lf/mg protein nitrogen (PN), 530 Lf/mg TN and 278 Lf/mg TN (used in subscript 2 toxoids) and saline; they were received in mid 1964. Toxoids $A_2$, $A_2S$, W, X and Y and saline Z were prepared by emulsifying aqueous toxoid or saline with an equal volume of an Arlacel A and mineral oil (Drakeol 6VR, Pennsylvania Refining Co.) adjuvant. The final volume of Arlacel A was 7.5%. Toxoids $B_1$, $B_2$ and $B_2S$ were made with an Arlacel A and 7-in-hexyloctadecane adjuvant(14).

Crude culture filtrates of *C. tetani* were prepared in this laboratory(15) and detoxified with 0.35% formalin. Emulsions were made by mixing equal volumes of crude toxoid (66 Lf/ml) with the Arlacel A and Drakeol 6VR adjuvant.

Five cholera vaccines were studied. Two were U. S. commercial products; one with an oil adjuvant had been used in a field trial in the Republic of the Philippines and was kindly supplied by Dr. H. Ogonuki(2,16); 2 were experimentally prepared by Dr. J. C. Feeley. One of the latter was phenol-killed and preserved and the other was formalin-killed and thimerosal preserved. Emulsions were prepared as with the experimental toxoid.

An emulsified typhoid vaccine and an emulsified trachoma vaccine were kindly supplied by Dr. S. D. Bell, Jr.(3). Four commercial typhoid and 2 typhoid and paratyphoid vaccines were used as well as several lots of influenza virus and adenovirus vaccines and allergenic extracts of insect antigen, house dust, mold, and ragweed prepared by several manufacturers.

*Emulsification of Arlacel A.* A 2% (v/v) emulsion of Arlacel A in water was prepared by sonic vibration (Sonifier, Branson Instruments, Inc.).

*Assay of fatty acid.* A modification(17) of the colorimetric method of Duncombe was used to assay the free fatty acid (FFA) content of a product. Phosphate buffer 0.025 M, pH 7.0, was added to an aliquot of the test material to a final volume of 1 ml. After addition of a copper nitrate-triethanolamine reagent and 4 ml of chloroform, the tube was shaken for at least 30 minutes. Following centrifugation the materials above the chloroform phase were removed and discarded. Two ml of the chloroform phase were added to a cuvette containing 0.2 ml of a 0.1% solution of sodium diethyl-dithiocarbamate in n-butanol. The optical density (OD) was read at 440 mμ (Coleman Junior Spectrophotometer) and the micromoles of FFA were calculated by use of a standard palmitic acid curve.

*Assay of hydrolytic activity.* To determine the effect of antigens on Arlacel A, 0.25 ml of the antigen to be tested was added to 1.0 ml of a 2% emulsion of Arlacel A in water. The mixture was incubated in a water bath at 37°C for 18 hours, then 0.5 ml was titrated for FFA. The time of incubation was varied for the tetanus filtrates. Portions of some of the antigens were heated in a water bath at 74°C for 1 hour. In addition, tetanus filtrates were heated at 36.4°C, 50°C, and 74°C for varying times. Heated samples were held in a water ice bath until all samples were ready for assay.

*Results. Free fatty acid content of emulsified antigens.* Table I presents the mean values of 2 to 19 replicate assays of FFA content of emulsified toxoids and vaccines used in field studies. Tetanus toxoid $B_2$, which induced abscess formation, contained approximately twice as much FFA as toxoid $B_1$, which did not induce abscess formation when administered 3 years prior to these assays. No toxoid $A_1$ was available for comparison, but toxoid $A_2$ had 1.7 times as much FFA as $B_1$. The adsorbed and plain toxoids contained no FFA. The cholera vaccine and the typhoid vaccine, both of which induced abscesses, contained amounts of FFA per dose comparable to that of $A_2$ toxoid.

A 0.5 ml dose of emulsified toxoid was

TABLE I. Free Fatty Acid Content of Antigens Used in Clinical Studies.

| Antigen | Mean FFA, micromole/single human dose |
|---|---|
| Tetanus toxoid, emulsified | |
| $A_2$ | 3.0 /.5 ml |
| $A_2S$ | 2.87/.5 " |
| $B_1$ | 1.66/.5 " |
| $B_2$ | 3.56/.5 " |
| $B_2S$ | 3.34/.5 " |
| Tetanus toxoid, adsorbed | |
| $C_1$ | 0 |
| $C_2$ | 0 |
| Tetanus toxoid, plain | |
| $D_1$ | 0 |
| $D_2$ | 0 |
| Cholera vaccine, emulsified* | 2.4 /.2 ml |
| Typhoid " " † | 2.35/.25 " |
| Trachoma " " † | .95/.25 " |

* Supplied by Dr. Ogonuki.
† Supplied by Dr. Bell.

calculated to contain 0.037 ml (7.5%) of Arlacel A. The assayed FFA content of 0.037 ml of the lot of Arlacel A incorporated in these products was 0.74 micromole. Therefore, the total FFA in the toxoid was not due to Arlacel A *per se*. Drakeol 6VR contained no detectable FFA. No 7-n-hexyloctadecane was available for assay.

Table II shows FFA content of a single 0.25 ml dose of toxoids W, X, Y and Z. The purer the parent toxoid the lower the FFA content of the emulsified toxoid. The differences in FFA in toxoids W, X and Y were significant at the 5% level. The difference in FFA content of W and Z was not significant. A 0.5 ml dose would have contained values comparable to those given in Table I.

*Effect of various biologics on an emulsion of Arlacel A.* Table III presents the FFA content of 0.5 ml assay samples of a 4 to 1 mixture of 2% Arlacel A in water and various biologic products (unheated and heated) after 18-hour incubation at 37°C. Heating at 74°C for 1 hour altered the ability of the reactive biologic to release FFA from the Arlacel A emulsion.

TABLE II. Free Fatty Acid Content of 4* Emulsified Tetanus Toxoid Preparations.

| Preparation | Specific activity of toxoid | FFA,† micromoles/.25 ml |
|---|---|---|
| W | 1370 Lf/mg PN | .64 |
| X | 570 Lf/mg TN | .92 |
| Y | 278 Lf/mg TN‡ | 1.14 |
| Z | None (saline) | .71 |

* Prepared with a common lot of Arlacel A and Drakeol 6VR.
† Mean of 5 replicate determinations.
‡ Same toxoid as was used in $A_2$-$D_2$ series.

TABLE III. Influence of Biologics on Release of FFA from Arlacel A During 18 Hours at 37°C.

| Biologic | FFA, micromole/sample* Unheated | 74°C for 1 hr | Difference unheated and heated |
|---|---|---|---|
| Bacterial preparations | | | |
| Filtrate *C. tetani*, BB | .95 | .17 | .78 |
| " " AA | 1.15 | | |
| Tetanus toxoid, U, exp† | .1 | | |
| Cholera vaccine, E | .24 | .09 | .15 |
| " " I | .58 | .14 | .44 |
| " " F, exp | .56 | .09 | .47 |
| " " P " | .65 | .09 | .56 |
| Typhoid vaccine, M | .21 | .14 | .07 |
| " " W | .1 | .1 | .0 |
| Viral preparations | | | |
| Influenza virus vaccine, 1 | .14 | .14 | .0 |
| " " " 2 | .16 | .15 | .01 |
| " " " 3 | .16 | .12 | .04 |
| Adenovirus vaccine | .14 | | |
| Allergenic extracts | | | |
| Stinging insect antigen | 1.09 | .21 | .88 |
| Special insect mixture | 1.17 | .60 | .57 |
| Wasp | .02 | .02 | .0 |
| House dust, 1 | .14 | .08 | .06 |
| " " 2 | .08 | .05 | .03 |
| " " 3 | .47 | .21 | .26 |
| Mold | .05 | | |
| Common and giant ragweed | .2 | .06 | .14 |
| Giant short western ragweed | .85 | .12 | .73 |
| Mixed ragweed | .07 | | |
| Controls | | | |
| Saline | .09 | | |
| Medium for *C. tetani* | .15 | | |

* Sample contained .1 ml biologic and .4 ml 2% Arlacel A (.008 ml Arlacel A).
† exp = experimental.

The *C. tetani* filtrates increased the FFA content of the mixture but the toxoids and the medium for growth of the *C. tetani* did not affect FFA content under the conditions of these experiments. Each of the cholera vaccines caused an increase in FFA. The activity of vaccine E was less than that of the other

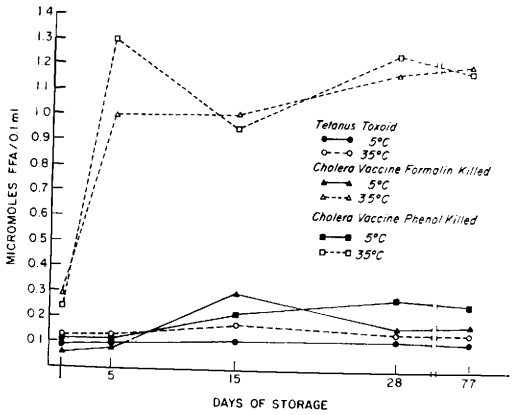



FIG. 1. Free fatty acid content of water-in-oil emulsions of tetanus toxoid and cholera vaccines stored at 5°C and 35°C for 77 days.

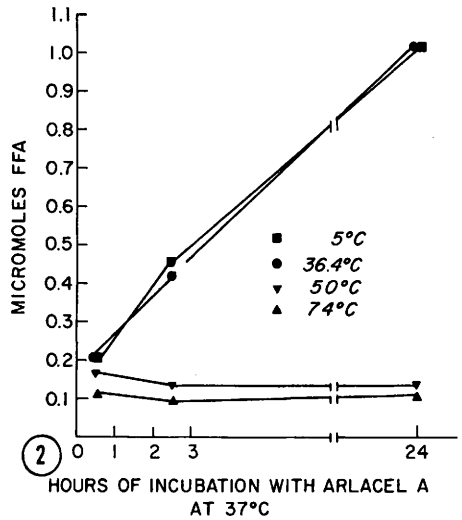
commercial vaccine I and the 2 experimental vaccines F and P. Commercial typhoid vaccine M appeared to have slight hydrolytic activity. However, 3 other typhoid vaccines, one listed in Table III, had no effect on the Arlacel A. No effect of 2 lots of typhoid and paratyphoid vaccine was observed.

The two types of viral vaccines did not release FFA from Arlacel A. A wide range of activity was detected within a single type and between different types of allergens. The activity of the allergens also varied in susceptibility to heating; the special insect mixture was most resistant. None of the biologic substances contained detectable FFA in the absence of Arlacel A.

*Effect of temperature and time on release of FFA by experimental tetanus toxoid and cholera vaccine emulsified in oil adjuvant.* Fig. 1 shows that FFA values of experimental emulsified tetanus toxoid stored from 1 to 77 days at 5°C and 35°C did not change significantly, although the emulsion of the toxoid stored at 35°C was broken. However, with cholera vaccines, either phenol-killed and preserved or formalin-killed and thimerosal preserved, there was at least a 10-fold increase in FFA with storage at 35°C. The vaccines stored at 5°C showed only a slight increase in FFA at the end of the 77 day period.

*Effect of temperature and time of incubation on hydrolytic activity of* C. tetani *filtrates.* Fig. 2 shows that the component in *C. tetani* cultures responsible for the release of FFA from Arlacel A was temperature sensitive. Heating the filtrate for 1 hour at 36.4°C prior to addition to 2% Arlacel A emulsion did not significantly affect the hydrolytic activity, but heating either at 50°C or 74°C markedly decreased this activity. The FFA content of the reaction mixtures containing 5°C and 36.4°C treated toxin was dependent on the time of incubation of the mixture.

Fig. 3 shows that loss of activity of the



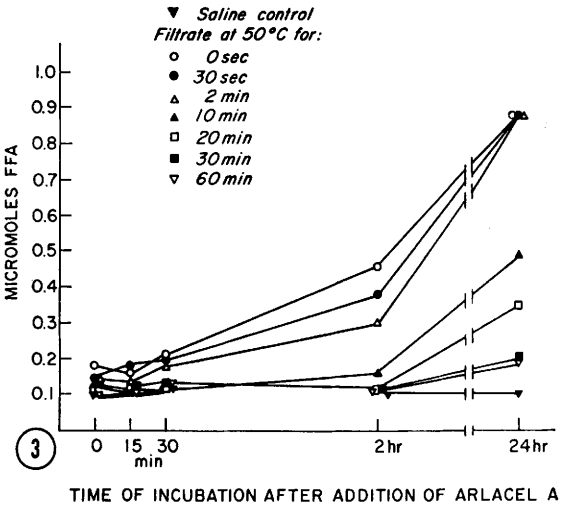




FIG. 2. Effect of *C. tetani* filtrate heated for one hour at 5°C, 36.4°C, 50°C and 74°C on release of FFA from Arlacel A at 37°C and rate of release.

FIG. 3. Effect of heat inactivation at 50°C on release of FFA from Arlacel A.

filtrate at 50°C was time dependent. The inactivation followed a first order process with a velocity constant of approximately 0.066 $min^{-1}$.

*Discussion.* It has been shown that antigens in oil adjuvant which caused abscess formation in human beings contained a higher FFA content than did similar antigens which caused no abscess formation. Such adverse reactions in recipients of influenza virus vaccine have been attributed to impurities(4), such as free oleic acid(11), of the emulsifying agent, Arlacel A. However, we have found that certain antigens are capable of releasing FFA from Arlacel A while others are not. It is of special interest that the influenza virus vaccines tested did not hydrolyze Arlacel A and that this antigen in oil adjuvants has been used extensively in the U. S. defense forces since improvement in the purity of the Arlacel A, and with very low incidence of local reactions(9-10). Recently a few reactions to influenza virus vaccine in oil adjuvant occurred in England(18).

The FFA content of 3 emulsified toxoids prepared simultaneously from common lots of Arlacel A and Drakeol 6VR was inversely correlated with the degree of purity of the parent toxoid. In addition, the FFA content of the 2 emulsified toxoids used in New Guinea was similarly related to the purity of the toxoid. Abscesses were seen in those women receiving the toxoid of lower purity and higher FFA content(1). Although toxic filtrates of *C. tetani* hydrolyzed Arlacel A, the toxoids prepared in our laboratory were inactive. However, the experimental toxoids were dissimilar to the commercially prepared products in methods of preparation, concentration, pH, storage, etc. The toxoids low in purity apparently contained an active component, similar to that in the toxic filtrates, possibly an esterase, which promoted the release of FFA from the Arlacel A. The relationship of the active component detected in the toxic filtrates to the esterase described by Imbriano(19) is under investigation.

Although inactivation of the experimental *C. tetani* filtrates with formalin destroyed the hydrolytic effect of the toxoid, neither formalin nor phenol altered the active component of the cholera vaccines. Cholera vaccines, both experimentally and commercially prepared, were very active in degrading Arlacel A; this activity is no doubt responsible for the increased FFA values detected in the emulsified cholera vaccines. This component may be related to the lipase of *Vibrio cholerae* described by Narayenan *et al*(20).

The wide variation in the activity of allergenic extracts and the small amount of antigen routinely administered in emulsions for hyposensitization might explain the low incidence of abscess formation(21) and yet explain why some abscesses occur, if FFA is related to induction of local reactions.

The relation of free fatty acid to abscess formation remains to be determined. Nevertheless the correlation of the presence of FFA in tetanus toxoids and certain vaccines with induction of abscess formation and the ability of certain antigens to hydrolyze Arlacel A indicate that caution must be used in selection of antigens to be incorporated in water-in-oil emulsions containing Arlacel A. Temperatures above 5°C which may occur under adverse conditions of storage and shipment may enhance the hydrolytic activity of these antigens. The presence of FFA *per se* may not be significant, but may accompany other as yet unidentified changes which are more directly related to the etiology of the local lesion. However, many workers(22-25) have reported the induction of various local tissue reactions with fatty acids. Strauss *et al*(26) recently reported that sebum and comedome suspensions containing FFA were capable of exciting an intense inflammatory infiltrate in human beings; the activity of the suspensions after FFA extraction was markedly decreased. Their preliminary data indicate that the length of the chain of the fatty acid is related to the degree of inflammatory response.

Further studies are needed to determine the ability of free fatty acids to induce inflammatory reactions and to correlate definitely the abscess formation of biologics in oil emulsions with the hydrolytic ability of the antigen on Arlacel A. Although not studied here, the role of hypersensitization to certain antigens such as penicillin(9) and pertussis vaccine(27) must not be neglected.

*Summary.* With use of the modified method of Duncombe for assay of FFA, it was found that water-in-oil emulsified tetanus toxoids, cholera and typhoid vaccines which induced abscesses in human beings contained more FFA than did emulsified products not inducing abscesses. The purer the parent tetanus toxoid, the lower was the free fatty acid content of the emulsified antigen. *C. tetani* culture filtrates, cholera vaccine, one typhoid vaccine and several types of allergenic extracts promoted the release of FFA from the emulsifier, Arlacel A. Preliminary data suggest that the hydrolytic component of *C. tetani* filtrates is an enzyme. The relationship of FFA released by the interaction of antigens on the emulsifying agent of the oil adjuvant to untoward reactivity remains to be determined.

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