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Project: Development of Immunodiagnosis and Improvement of Immunotherapy of Snake Venom Poisoning

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Progress Report

By

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Activity #1: Immunological characterization of venom proteins: relative abundance, immunogenicity and antigenic relatedness.

This objective has been almost accomplished during the first six months of this project. The experiments and results can be briefly summarized as follows:

1.1 Molecular sizes and relative abundance of venom proteins of the six Thai poisonous snakes.

With the use of polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulfate (SDS), proteins of each snake venom can be separated according to their sizes. These proteins are visualized by staining them with Coomassie blue. Since the amount of dye bound to the protein is roughly proportional to the protein content in each band, the intensity of the blue color reflects the relative abundance of the protein in the venom. The results of this study are representatively shown in Fig. 1.

Among the elapids, *N.n. siamensis* venom shows the highest abundancy of low MW components, mostly postsynaptic neurotoxin of about 7,000-8,000 daltons. *B. fasciatus* and *O. hannah* venoms give more high MW venom proteins while the contents of 7,000-8,000 daltons proteins are relatively less. This is in accordance with the lower lethality of these two venoms as compared to that of *N.n. siamensis* venom. One interesting observation as far as the viperid venoms are concerned is that they contain a lot of proteins of MW less than 10,000. Since most, if not all, of the known toxic components of these venoms, as well as

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all known enzymes are of MW greater than 10,000 daltons, it is intriging as to what the functions of these proteins are.

The results obtained from SDS-PAGE study will allow us to characterize the venoms immunologically (e.g. using Western Blot as reported below) and biochemically during purifications of the major toxins of these venoms.

1.2 Immunogenicity and antigenic relatedness

Under electrophoresis, protein bands on the SDS-polyacrylamide gel can be transferred to a sheet of nitrocellulose paper (Western Blot). The nitrocellulose paper with venom proteins on it can be treated with rabbit homologous antibody or heterologous antibody. The extent of binding of the homologous antibody to each venom protein band represents the relative immunogenicity of the venom protein. Binding of heterologous antibody, however, represents the degree of immunological relatedness or cross-reactivity.

Experiments on this aspect have been carried out using all possible combinations of snake venoms and rabbit antibodies. The results on the relative immunogenicity of venom proteins is shown representatively using *O. hannah* venom and its corresponding rabbit antibody in Fig. 2. From these results, the following conclusions are made or confirmed:

Immunogenicity

a) Low MW toxins (MW 7,000-8,000) are relatively less immunogenic than other venom proteins. In the case of *O. hannah* venom (Fig. 2) for example, almost no antibody binds to the post-synaptic neurotoxin region. This explains the difficulty in producing high potency antivenin against most elapid venoms.

b) As expected, the high MW venom components are more immunogenic. In Fig. 2, extremely immunogenic proteins of about 23-25,000 daltons in *O. hannah* venom are observed. It will be interesting to see whether the neurotoxins can be made more immunogenic by chemically coupling them to these proteins.

Immunochemical cross-reactivity

A representative result of the Western Blot of the six Thai snake venoms stained with rabbit antiserum produced against O. hannah venom is shown in Fig. 3.

a) Immunochemical cross-reactivity is observed among venom proteins of elapids or among those of viperids. However, inter-family cross reactivity is detectable, but is not as extenssive as that within a family. For example, a few venom proteins of N.n. siamensis venom react with rabbit antibody produced against Trimeresurus venom. The cross-reactivity observed here may present a problem in our attempt to develop on immunodiagnostic kit for detection of snake venom.

b) Immunochemical cross-reactivity between elapid toxins is absent or minimal. This is evident in the Western Blot in the region of MW 7,000-8,000 daltons. This explains the long observed lack of cross-neutralization between antivenins produced against elapid venoms.

Activity # 2. Purification of elapid principle postsynaptic neurotoxins.

Principle postsynaptic neurotoxins of elapids i.e. N.n. siamensis, B. fasciatus and O. hannah venoms are needed for the following studies

a. Immunochemical analysis of these toxins using monoclonal antibodies.

b. The study of relative immunogenicity of various forms and preparations of these toxins in experimental animals. These aim at the production of more potent or cross-nentralizing antivenins.

c. The development of immunoassay for in vitro determination of antivenin potency.

The progress made on this aspect of study is as follows:-

1.1 The principle postsynaptic neurotoxin of N.n. siamensis venom (the siamensis toxin 3) has been purified and is available in hundreds of milligrams quantity.

1.2 The principle postsynaptic neurotoxin of B. fasciatus venom (the B.fasciatus toxin IX) has been purified, between only a few milligrams is available due to the low abundancy and poor recovery.

More chromatographic runs will have to be made to accumulate enough toxin for this project.

1.3 Attempts at purifying the postsynaptic toxins of O. hannah venom (claimed to be two and called toxins a and b) have not been successful. We are still trying to do this and hope to accomplish this in the near future.

Activity #3. Development of Immunodiagnostic kits. The purpose of this study is to develop rapid, simple immunoassays to detect various snake venom proteins for rapid diagnosis of the envenomating snake. Two techniques have been explored so far. These are hemagglutination inhibition using either sheep or turkey erythrocytes and latex agglutination. Rabbit antibody prepared against the six Thai poisonous snake venoms are used. These antibodies are used at various states of purity: immunoglubulins obtained from ammonium sulfate precipitation, immunoglobulins from DEAE-cellulose chromatography and finally those obtained from chromatography on Staphylococcal protein A-Sepharose affinity columns.

Using passive hemagglutination by coating erythrocytes with each of the snake venoms, the 'sensitized' erythrocytes could be agglutinated in the presence of antibody against the snake venom. This reaction can be inhibited when free, soluble snake venom (the one used to coat the cells) is added, the so called hemagglutination inhibition reaction. Using this technique conditions have been optimized so that the detection of snake venoms can be made with the sensitivity of the assay shown in Table 1.

By adding heterologous antigen ie. other snake venoms into the hemagglutination reaction, inhibition can occur if the added venom cross-reacts with the antibody. This type of cross-reactivity can cause ambiguity in the test. The assay described here showed little cross-reaction and only at very high concentration of the cross-reacting venom. (Table 1.).

It should be mentioned that the sensitivity of this test is relativly low when compared to that of RIA and ELISA. However, it should be sensitive enough since venom collected from a patient wound would usually far exceed the detection limit.

Under study is the possible interference of various body fluids, i.e. plasma, serum, lymph, etc. on this test.

Attempts are being made to develop a latex agglutination test similar to the one explained above for hemagglutination. However, optimal conditions for the test have not been completely worked out.

Activty #4. Production of monoclonal antibody

In terms of the production of monoclonal antibodies against the principle Elapid toxins, our aims during the initial period of this project were as follows:

1. to acquire the essential components for hybridoma production, screening and cultivation.

2. to begin the immunization of mice with purified principle toxins (*Naja naja siamensis* toxin 3 selected as prototype).

2.1 to adapt a suitable assay for monitoring the response of animals immunized by different regimens

2.2 to select from among the various immunization schemes used, those stimulating the best response

2.3 to develop a method of detoxifying the antigen enabling high-dose injections without adjuvant immediately prior to fusion, preferably preserving the native structure so as to make pre-fusion booster(s) compatible with earlier priming and secondary immunizations.

3. to develop or adapt an appropriate assay suitable for the rapid screening of large numbers of hybridomas, in order to select the relatively few secreting monoclonal antibodies against the toxin.

4. to begin making fusions between immune mouse spleen cells and mouse myeloma cells.

Materials and Equipment:

Sufficient tissue culture plastic ware and ELISA plates have been ordered and received to support hydridoma production for the first 6-9

months. Most of the reagents for hybridoma production have been obtained. One limiting factor which remains is the selection of a good lot of the fusogen, polyethylene glycol. Six lots have been obtained of which 4 were found unacceptably toxic at a 40% concentration; one was marginal in terms of toxicity, but did not give good fusion. The batch remaining to be tested consists of commercial "pre-tested tissue culture grade", but is available in amounts too small to allow the usual in house pre-screening for toxicity/fusability and will have to be judged only by the end result - the number of hybridomas. Providing that this material gives good reproducible fusion, it should bypass the need for additional screening.

A second related though not as severe problem is that the foetal bovine serum (FBS) we are now using will not alone support clonal growth, (i.e. single-cell or low density growth) of the 653 myeloma culture cells (it partially supports the less fastidious Sp2/0 line). Unfortunately, the yearly demands of our laboratory are not sufficient to entice suppliers to provide batch samples for testing with the option of reserving or purchasing large amounts of the same lot. We are attempting to negotiate with other laboratories maintaining the same or similar lines to arrange pooled orders which collectively would exceed supplier-set limits and enable aliquot pre-testing.

Major support equipment which has not yet arrived has presented only minor problems during the initial stage where conditions for fusions and design of assays are being worked out and time is being invested in technician/graduate student training and in immunization. This material should arrive before hybridoma production is in full scale.

Immunization:

Any immunization procedure must take into account the high toxicity of the native venom toxins as well as the low molecular weight and consequent low immunogenicity. We had initially proposed to use Sepharose-coupled toxin as immunogen under the assumption that the coupling would immobilize the toxin thus effecting de-toxification and enabling priming with amounts well above immunogenic thresholds. This material has proven unmanageable in early attempts as it settles too

rapidly and clogs syringe needles preventing an accurate dose delivery. We also found that at reasonably high doses, a least the CNBr- coupled material was not sufficiently detoxified, presumably as a result of leaching. We may later reinvestigate this area by increasing the viscosity and perhaps using a more stable method of covalent attachment, however, conventional immnizations in increasing doses employing mineral oil adjuvants have given reasonable responses in roughly half of the mice. Thus, despite loss of animals, we have relied upon the "depot effect" of Freund's adjuvants to provide sufficient detoxification, while enabling "native" antigen to be administered.

An unanticipated setbeck was enountered in our earliest immunizations when an unidentified contageous disease rapidly swept through the immune mouse colony infecting all mice. This disaster, which preceded any fusions, set us back 3 months, however, additonal precautions and restrictions have been applied to prevent its recurrence. Despite the fact that our various immunization procedures using Freund's complete and incomplete adjuvant were done on small (non-statistical) groups of animals, we expected to see general patterns of effectiveness in terms of priming doses, fold-increases of booster doses as well as timing or spacing of injections. Such a pattern does not seem to be clearly emerging at present and indications are that it would be apparent only if immunizations were done on a larger scale using more readily available outbred mice. Individual variation is apparently masking any such general pattern. Our major concern at present, however, is to obtain at least a few monoclonal antibodies to begin characterizing, rather than to determine the optimal immunization scheme, and perhaps this individual variation will increase the diversity in terms of epitopes and affinities of monoclonal antibodies resulting from different fusions.

We have been concerned about finding a method of adminitering reasonably high doses of antigen in soluble form at the "pre-fusion booster" stage 3 days before fusion. It must be soluble if it is to reach the spleen cells in time to stimulate cell division and is effective in proportion to dose, hence it must be detoxified. Our preferred method was to detoxify by covalent attachment to a soluble

carrier protein and to a different carrier than might be used in priming immunizations. The inital attempts to couple toxin to succinylated BSA using the water-soluble carbodiimide EDAC have not given a sufficiently high coupling ratio. This carrier was selected in the hope that NH_2-group modified carrier would force the crosslinkage through the added COOH groups of BSA and the lysine groups of the highly basic toxin, minimizing homo-polymerization of either. Our ratios of toxin: carrier: crosslinker, volumes and times of reaction, selected from ranges provided in the literature need to be better adapted to this particular system. The choice of a succinylated carrier is causing difficulties in the subsequent immunological assay presumably through nonspecific electrostatic interactions between an acidic carrier and more basic antibodies. This area definitely needs further investigation and system optimization using more standard carriers (KLH, thyroglobulin, albumins, etc.), since at present we are using partially self-polymerized toxin for pre-fusion boosters. This glutaraldehyde-reacted toxin is sufficiently detoxified to enable high doses, however, the native structure has undoubtedly been altered. We are concerned that only a subset of the cells stimulated in the initial priming immunizations will respond in the important pre-fusion booster and hybridoma recovery will suffer and/or some of the monoclonal antibodies will no longer react fully with the native toxin. The glutaraldehyde-treated toxin is, however, only a temporary substituts until we can obtain a more ideal immunogen.

Antibody Assay:

An appropriate assay was needed to monitor the immune response in mice in order to follow the effectiveness of booster doses and allow us to tailor the immunization to individual mice more carefully, thereby avoiding loss of valuable animals through toxin overdose. Perhaps more importantly, an assay was needed in order to select the single best responder from among several immunized by the same or similar regimen and the most suitable time for performing the fusion. There are no time restrictions and the number of sera to be compared is small, hence almost any conventional antibody assay should work. A second point where an antibody assay is needed is for the initial screening of

hydribomas. Unlike the assay used to follow the mouse response, which could be a lengthy, exact procedure indicating which protein and to what titer a response is made, the hybridoma screen must accomodate large numbers (perhaps 1000 or more) and must be rapid (1 or 2 days at most). These restrictions favored a solid-phase antigen enzyme immunoassay which could be patterned after the 96-well hybridoma culture plates. We anticipated that the extreme basicity and low MW might complicate adaption of such an assay in that either the toxin might not adhere well to plastic or might be sterically blocked by a larger protein (e.g. BSA) used to saturate and prevent non-specific Ab binding. Fortunately, the assay worked extremely well with either a detergent- or protein saturant and gave undetectable non-specific binding. Since our immunogen is essentially a single protein species, the same microtiter plate assay has been quite suitable for mouse screening in addition to monoclonal antibody assay. By this assay, the 2 mice used in fusions achieved reciprocal dilution titers in excess of 51,000 — but only by about 4 months of gradually increasing booster doses. As anticipated, our results indicate that the low MW toxin is not a good immunogen, but other mice are responding albeit more slowly. We have not as yet correlated loss of mice through toxin booster overdose with low range titers, but have not observed loss of animals with 4-5 fold increasing doses once reciprocal titers of 800-1600 have been obtained.

Fusion:

As indicated above, we have performed only 2 fusions at present and both used high-response mice. Both fusions were lost to bacterial contamination which was traced to filter-sterilized "conditioned media" added to offset the suboptimal growth stimulating properties of non-pre-selected FBS. The presumably antibiotic-resistant bacteria were not identified however this source should be eliminated with the use of 0.2 micron filters.Our selection of the PEG fusogen has much room for improvement and hopefully, the "tissue culture tested" grade will work well and provide a reliable and consistent source. As mentioned, the 5g ampoules supplied do not allow sufficient for any pretest other than simply performing the fusion and optimizing concentrations in subsequent fusions. Should selection of the PEG remain a problem, non-immune or

poorly responding mice will be used to assess fusability rather than to waste our best responders. We have been spacing our priming immunizations of different batches of BALB/c mice following the colony disaster so as to provide a continuous supply of immune mice beyond this point and we are continually administering increasing dose boosters to the poorer responders.

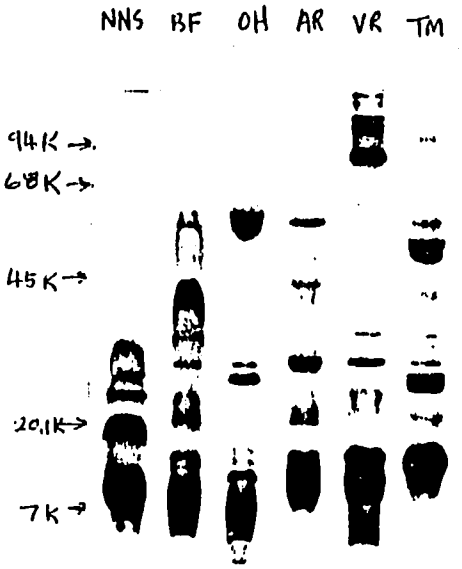

Fig. 1. SDS polyacrylamide gel electrophoresis of the six Thai snake venoms.

Fig. 2. Western Blot of O. hannah venom on nitrocellulose paper. The right hand lane was stained with Amido Schwartz. The left hand lane was stained with ~~of~~ rabbit homologous antibody followed by enzyme conjugate.

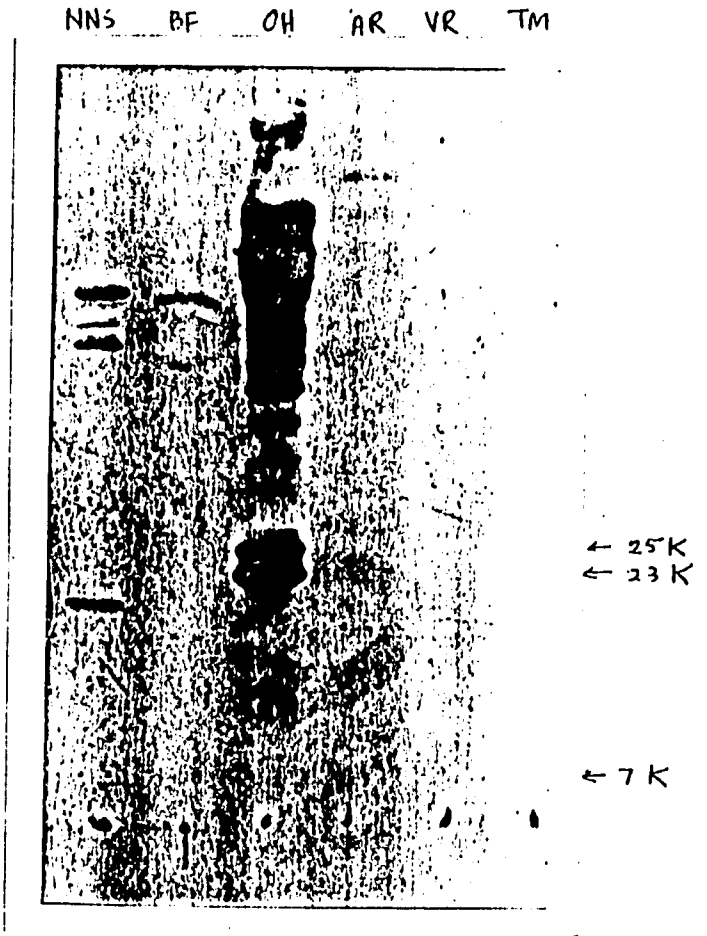

Fig. 3. Immunochemical cross-reactivity between various snake venom proteins. The figure shows the Western Blot of the six snake venoms stained with rabbit antibody raised against O. hannah venom.

Table 1. Sensitivity of detection and cross reactivity of venoms in passive hemagglutination inhibition assays.

Venom	Antibody to venom	Detection limit µg/well	Cross reacting venom (µg/well)					
			Nns	Bf	Oh	Ar	Vr	Tp/a
N.n. siamensis	IgG 123	5.62		–	>800	–	–	>1600
	Serum 125	5.62						
B. fasciatus	IgG 241	0.72	–		>1600	–	–	>1600
	Serum 241	1.45						
O. hannah	IgG 612	4.06	–	–		–	–	>1600
	Serum 612	4.06						
A. rhodostoma	IgG 361	8.12	–	–	–		–	–
	Serum 365	8.12						
V. russelli	IgG 484	0.51	–	–	–	–		–
	Serum 484	0.51						
T. popeorum/ albolabis	IgG 511	0.39	[illegible]					
	Serum 511	0.78						