EXPERIMENTAL STUDIES OF INDUCING AGENTS OF GUINEA PIG LIVER CHANGED BY IMMUNOLOGICAL TECHNIQUE

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INTRODUCTION

In 1934, Holtfreter[1] demonstrated that different adult tissues of *Triton* showed regional specificity in the inductive effect on amphibian gastrula. After Toivonen[2,3] examined the regionality in induction by vertebrates' tissues, he postulated that the induction mechanism of these tissues might be the same as or at least similar to the process of normal development, and that these tissues might contain some special inductive substances. At the same time, Chuang[4,5,6] used mouse kidney and *Triton* liver either in the fresh state or after being boiled as inductors in implantation and explantation experiments on *Triton* gastrula. He showed that the inductive agents of mesodermal structures changed to neural, and finally vanished, by prolonged heat treatment of the inductors. In addition, it has been observed that the alteration of the inductive agent occurred in organizer or other tissues not only after heat treatment, but also by other agents such as formol or organic solvents (Vahs[7,8,9], Kawakami & Mifune[10], Mifune[11], Engländer & Johnen[12]). Other investigators postulated that some implanted tissues or substances exert toxic effect on the surrounding host cells to release an active agent (Barth & Graff[13], Okada[14], Okada & Hama[15]). This idea was advanced by Holtfreter[16,17] through his experiments, in which the presumptive ectoderm of gastrula was neuralized by subjecting it to acidic or basic media under sub-lethal conditions. It was also supported by the dorsalization or activation of the ventral marginal zone of *Triturus* gastrula with the similar treatment (Yamada[18], Kawakami & Okano[19]).

By noting that neuralization of the presumptive neural plate could be affected by using reductive or oxidative reagents, Brachet[20] suggested that proteins which were denatured by heat or other agents would also participate in induction phenomena. Lehmann[21] speculated that the spino-caudal inductive factor(s) might be protein, and that the archencephalic inductive factor(s) might be related to nucleic acids or their decomposed products. That inductive substances have some relation to proteins was supported by Toivonen & Kuusi[22], Hayashi[23], Takata[24] and Vahs[9]. They showed that inductive ability vanished after treatment with proteolytic enzymes such as pepsin or trypsin, and that the tissues or their pentosenucleoprotein did not

show any modification in their inducing properties after treatment with ribonuclease.

Yamada and his co-workers (Yamada[25], Yamada, Takata & Osawa[26], Yamada & Takata[27,28], Hayashi[23,29,30], Yamada[31,32], Takata[24]) reported that proteins, obtained by precipitating with ethanol from the isotonic saline extracts of liver, kidney or bone marrow of guinea pig, showed the same regional induction as those of the original tissues. Afterwards, they obtained a pentose nucleoprotein fraction from the isotonic saline extracts of liver tissue by ultracentrifugation as a non-sedimentable subfraction, and showed that the specific inductive effect of liver was comparable with that of this subfraction. Kawakami and Iyeiri[33] were able to isolate the mesodermal and neural inductive agents from the 1/3 saturated ammonium sulfate salted-out protein of chick embryo extract by chromatography on a column of hydroxylapatite.

From the stand-point of "molecular ecology", Weiss[34] postulated that there would be some interaction between an inductor and an adjacent layer, and that "the inductor itself would operate only on the surface of the latter a steric conformance, i.e., complementary spatial atomic groups of them enabling them to conjugate in key-lock fashion".

Some investigators (Kritshevsky[35], Idzumi[36], Ten Cate[37]) confirmed by immunological methods that the antigens in embryo altered qualitatively and quantitatively during development and suggested that chemical differentiation of substances would take place prior to observable morphological changes. Clayton[38] was able to demonstrate by absorption methods specific mesodermal and ectodermal antigens in addition to common antigens during the gastrulation, neurulation and tailbud stages of *Triturus alpestris*. According to Vainio[39], there are antigenic differences between the blastoporal lip and the presumptive ectoderm in *Triton* gastrula. He further states that the blastoporal lip contains the same antigenic factors as bone marrow since the latter cross-reacted with antiserum prepared against the soluble fraction of the former.

From experiments concerned with the temporal and spatial gradients of immunochemical radicals, Ishikawa[40] emphasized the significance of surface radicals in the development of the embryo, which has a gradual change both in basic substances and in surface radicals.

Of particular interest is the relationship between inductive and immunologic active sites; namely, whether the neuralizing inductive agents are altered by formation of antigen-antibody complexes. In the present communication, antisera against saline extracts of guinea pig liver were reacted with homologous antigen and the inductive effect of their antigen-antibody system was examined on the gastrula stage of *Triturus*.

MATERIALS AND METHODS

1. Preparation of Antigen

Guinea pigs of about 6 months old were used. Perfused liver was frozen, homogenized in a Potter-Elvehjem homogenizer for 3 min with 4 volumes of 0.14 *M* NaCl and centrifuged at 3,000 rpm for 15 min. The supernatant was precipitated at pH 4.2 by addition of acetic acid. This precipitate is designated as "low speed protein". The precipitate was redissolved in 0.14 *M* NaCl and was used as the antigen for immunization purposes and for the precipitin reaction.

In another experiment, the supernatant at 3,000 rpm was recentrifuged at 19,000 rpm for 15 min. The final supernatant was acidified (pH 4.2) and the precipitate which was used as antigen is designated as "high speed protein".

Some of the antigen solutions were lyophilized and kept in a dessicator until used as grafts or for the immunological reaction.

2. Immunizing Procedure

Immunization was carried out intravenously with the antigen only or intramuscularly with that emulsified with modified Freund's adjuvant. Five rabbits were used for "low speed protein" as antigen, and two rabbits for "high speed protein". Injections were repeated until a sufficient amount of each antibody was obtained as measured by precipitin ring tests.

3. Preparation of Antigen-Antibody Complexes as Inductors

Immunized rabbits were bled 5 to 20 days after the last injection. The titers of antisera were assayed by the ring test after heating for 30 min at 55°C to inactivate compliment. The equivalence point was determined to be that ratio of antisera to antigen which flocculated in the shortest time. Equivalent amounts of antigen were then added to the antisera and the antigen-antibody precipitate was collected and washed several times with Holtfreter's solution. The precipitate was suspended in 70% alcohol and was kept in an ice-box for one day. It was washed several times with sterile distilled water and Holtfreter's solution before its use in the subsequent experiments.

4. Explantation Experiments

The embryos of *Triturus pyrrhogaster* at the middle or late gastrula stage (St. 12 b-c, according to Okada & Ichikawa's normal developmental stage of *Triturus pyrrhogaster*) were used as donors of presumptive ectoderm. The presumptive epidermis was carefully excised from the gastrula, without including the notochord and mesodermal regions, and the inductor was wrapped with the presumptive epidermis.

The experiments are divided into 3 series. The 1st deals with the inductive effects of antigen, the 2nd with antisera, and the 3rd with those of the antigen-antibody complex.

1st experimental series: The lyophilized liver "low speed protein" (Ia) and "high speed protein" (Ib) were dissolved with distilled water and were precipitated with 70% alcohol (final concentration). The precipitated proteins were washed with

sterilized Holtfreter's solution and used as grafts.

2nd experimental series: Alcohol (final concentration, 70%) was added to the antisera against "low speed protein" (IIa & b) or "high speed protein" (IIc). The protein obtained was completely soluble in Holtfreter's solution, and unsuitable as the grafts. The mixture, therefore, was centrifuged at 3,000 rpm after being heated at 48°C for 5 min. Then the precipitate was used as the grafts after being kept in 70% alcohol for 1 day and washed with sterilized Holtfreter's solution.

3rd experimental series: The grafts were prepared by a precipitin reaction between "low speed protein" (IIIa~c) or "high speed protein" (IIId) and antisera against "low speed protein", or between "high speed protein" and its antiserum (IIIe).

As the control for all experiments, the presumptive epidermis without any graft was cultured in Holtfreter's solution in the same way as the experimental groups. It was tested to determine if the epidermis which was isolated contained any other organ-forming areas. The presumptive epidermis of the control group became a rigid and rugged explant 2 or 3 days after operation. None of the 18 control explants which were examined microscopically showed any special structure and they differentiated into an irregular epidermis. Thus, it was assumed that the isolation of the presumptive epidermis used in these present experiments as a reactor was done with no other organ-forming area present in the explant. These explants were fixed with Bouin's fluid about 12 days after the operation and stained totally with borax-carmine. After sectioning at 8μ, they were stained with "Blau Schwarz" for microscopic observation.

RESULTS

Inductive effects of antigen: The results are summarized in Table 1. In the experiments where the grafts were the lyophilized "low speed protein" (Ia), the tendency of induction was similar to that of liver tissue itself or of the liver fractions reported by Toivonen[3], or Kawakami and Yamana[4]. The frequency of induction was, however, rather lower compared to that obtained by using non-lyophilized material. In the case of "high speed protein" (Ib), a similar tendency with that of "low speed protein" was observed (Table 1). All these inductions were archencephalic in nature (plate 1).

Inductive effects of antisera: The grafts were rabbit antisera against "low speed protein" (IIa & b) and "high speed protein" (IIc), and were precipitated by ethanol and heating*. The frequency of induction of this series was generally very low, and the induction was poor in the differentiation of induced structures (Table 2).

* One part of the precipitated antiserum protein was dissolved in veronal buffer (pH 8.1) for the Tisellius electrophoresis. All components of the antiserum were found in the dissolved proteins and the amount of γ-globulin was not very different from that in the antiserum which was used in precipitin reaction.

Table 1 1st Experimental Series
Inductive effects of the lyophilized liver low[1] or high[2] speed protein.

Exp. No.	Ia	Ib
Kinds of Grafts	Low Speed	High Speed
Total	11[3]	46[3]
No induction	5	21
Archencephalon	3	8
Unspecific brain	2	3
Nose	1	1
Eye		5
Lens		1
Neural fragment	4	17
Mesenchyme	1	2

1): The liver protein, precipitated with isoelectric point after centrifuging at low speed, was dissolved in neutral 0.14 *M* NaCl, and lyophilized.
2): The liver protein, precipitated with isoelectric point after centrifuging at high speed, was dissolved in neutral 0.14 *M* NaCl, and lyophilized.
3): Some explants induced not only one structure, but also several ones, so that the total number of induced structures was over that of total explants operated.

Table 2 2nd Experimental Series
Inductive effects of the protein obtained from antiserum against liver "low speed protein" or "high speed protein" by adding ethanol and heating (48°C, 5 min).

Exp. No.	IIa	IIb	IIc
Kinds of Antigen	Low Speed	Low Speed	High Speed
Total	56	32	37
No induction	50	29	32
Neural fragment		1	1
Mesenchyme	4		1
Blood cell-like elem.[1]	1		
Undiff. mesoderm-like[2]	1	2	3

1): Abbreviation of Blood cell-like element.
2): Abbreviation of Undifferentiated mesoderm-like cell mass.

In IIa mesenchymal differentiation was shown in 4 cases, but it was ill-defined. Blood cell-like elements were observed in IIa, but these were very few in number. The tendency of induction in IIc was similar to that in IIa and b, but an undifferentiated mesoderm-like cell mass was shown in 3 out of 37 explants and a neural fragment was observed in but one explant.*

* It has been obvious that the alcohol treatment of grafts would change the inducing ability from mesodermal induction to neural one. When the grafts in IIc were soaked in alcohol for 5 days, neural structures such as unspecific brain, sensory organs and/or neural fragment were observed beside few undifferentiated mesoderm-like cell mass. These induced structures were usually organized.

Inductive effects of antigen-antibody complexes: This series consisted of 3 experimental groups. Two of these groups differed from each other in the antigen used for precipitin reaction with antisera against "low speed protein"; that is, "low speed protein" (IIIa-c) and "high speed protein" (IIId). The third group consisted of "high speed protein" and its antiserum (IIIe). The results are shown in Table 3. Some explants of one of the groups (IIIa) showed a bulgy form 2 or 3 days after operation. In some explants, both dispersed and contracted melanophores were especially observed. Other groups (IIIb & c) were irregular or round in form. In group IIId, the external forms of these explants did not show any particular type, but irregular and rugged shapes. By microscopical investigation, 7 notochords and 1 muscle were observed in 32 explants (IIIa) as shown in Table 3. One explant produced two notochords, deuterencephalon, and mesenchyme-cells (plate 2). In other explants, induced muscle and a long notochord surrounded by muscle were

Table 3 3rd Experimental Series

Inductive effects of the precipitate which were formed by precipitin reaction between "low or high speed protein" and its antiserum or between "high speed protein" and antiserum against "low speed protein".

Exp. No.	IIIa	IIIb	IIIc	IIId	IIIe
Kinds of Grafts	Low-Low[1)]	Low-Low[1)]	Low-Low[1)]	High-Low[1)]	High-High[1)]
Total	32[2)]	15[2)]	76[2)]	40[2)]	55[2)]
No induction	10	11	70	39	48
Archencephalon	10	2	1		
Unspecific brain		1			
Nose	1				
Eye	2				
Deuterencephalon	8				
Ear	8				
Neural fragment	2		2		3
Melanophore	1				1
Mesothelium			1	1	
Notochord	7				
Muscle	1	1?			
Mesenchyme	4	2	1		1
Blood cell-like elem.[3)]			1?		
Undiff. mesoderm-like[4)]					2

1): Abbreviation about the kinds of antigen-antibody complex, in which High-Low is, for example, that antigen is "high speed protein" and antiserum is the one against "low speed protein".

2): Some explants induced not only one structure, but also several ones, so that the total number of induced structures was over that of total explants operated.

3): Abbreviation of Blood cell-like element.

4): Abbreviation of Undifferentiated mesoderm-like cell mass.

observed (plate 3). Still another explant showed a notochord near the graft and a deuterencephalon was also observed nearby (plate 4). However, most of explants showed no induced structures or very poor differentiation.

In the group of "high speed protein" (IIId) as the precipitin antigen, the frequency of induction was quite low. No archencephalic structure was induced, and no induction of cephalic structures such as neural vesicle and neural cell-mass was observed. In some explants, masses of mesoderm-like cells were produced, which were classified as "undifferentiated mesodermal". The inductive effect of antigen-antibody complex prepared from "high speed protein" and its antiserum (IIIe) was rather stronger than that of the antigen-antibody complex of "high speed protein" and "low speed protein" antisera (IIId), but the inductive tendency of the former was similar to that of the others except of IIIa. The induced differentiation consisted of 3 neural fragments, 1 melanophore, 1 blood cell-like element and 2 undifferentiated mesoderm-like cell masses.

DISCUSSION

When liver protein, which is a well known neural inducing agent, was used as inductor after combining with the antiserum against it, it was found in the present experiments that the original inductive activity of liver protein was generally depressed by the antigen-antibody complex. It is known that the inducing capacities of various mammalian tissues are affected by various agents such as heat and organic solvents. Yamada and co-workers suggested that the mesodermal agent might be changed to a neuralizing one as the result of a denaturation process and that the mesodermal agent may be a thermolabile protein of large molecular weight (Yamada [25], Yamada & Takata[27,28], Yamada, Takata & Osawa[26], Hayashi[42]). According to Toivonen et al. the neuralizing agent, which exists in guinea pig liver, is relatively thermostable and is more dialyzable from the tissue than the mesodermal agent (Toivonen & Kuusi[22], Kuusi[43,44], Toivonen & Saxén[45,46]).

Piecing these considerations together with the results of the 3rd experimental series, the possibility existed that the archencephalic inductive effect of liver protein is modified to a mesodermal or spinocaudal type when the liver protein is combined with its antibody. However, most of the results in this experiment did not support this possibility, and there is some doubt whether the explants in IIIa which were isolated were free from materials of the marginal zone. Another possibility to explain this experimental result may have as its basis some immunological differences of antibody caused by the duration of immunization and other factors.

The explantation experiment, however, was carried out as carefully as possible and the isolated presumptive epidermis in the control experiment did not produce any structures other than irregular epidermis. Moreover, the notochords in IIIa were found around grafts, suggesting the notochord-inducing action of the grafts.

These points might deny the possibility of simultaneous explantation of marginal zone.

Burnet[47] suggested that immunological activity was related to the immunizing technique. It might be considered that the antigen for immunization in the present experiments contained various kinds of protein, and that the duration of the immunization differed in some cases. Moreover, Farr[43] suggested that the quality of antibody was quite variable from animal to animal. These points might explain the above difference in the 3rd experimental series.

Vainio[49] reported on the inducing effect of tissue inductors which were treated with rabbit antiserum. According to his results, the guinea pig liver tissue, which had been treated with rabbit antiserum prepared against the saline-soluble fraction of liver, induced predominantly mesodermal structures and a few archencephalic ones. At the same time, he stated that the inducing effect of implants was often inhibited when liver tissue treated with antiserum was used as inductor. These results harmonize with the results obtained in the 3rd experimental series, in which the inducing effect seemed to be depressed generally although the induced mesodermal structures were very rare in occurrence.

Vainio also tried to treat the tissue with normal serum. In his experiment with liver tissues, any difference in inducing ability between normal and immune sera was negligible. Therefore, he argued that the appearance of mesodermal induction was caused by the treatment with serum regardless of whether normal or immune sera were used.

The explantation of antiserum itself in the present experiment showed an archencephalic inductive effect, though of low frequency. As stated above, the serum in alcohol was coagulated with heat in a water-bath at 48°C. Therefore, the existence of inducing ability in serum might be suggested from these experiments.

In some cases in the present experiments, the antigen was lyophilized. Haudaroy and Tauner[50] reported the effect of lyophilization on the immunological activity of bacteria, demonstrating that the activity was preserved for one year at room temperature and for 30 days in a water-bath at 50°C. Even after boiling for 10 min at 100°C, the activity was not destroyed. The neural inductive capacity is resistant to heating below 100°C at least and to other various treatments such as with organic solvents as mentioned above. Liver lyophilized proteins obtained by centrifuging with either high or low speed both induced neural structures as did the newly prepared non-lyophilized protein. The same induction results were found with both "high" and "low speed protein" of liver.

With the exception of mesodermal inductions obtained in the 3rd experimental series, the depressive action of the antiserum on the inductive capacity of the antigen is worthy of notice. A logical conclusion from this fact is that the immunologically active radical of antigen is the active radical of the inductive agent itself, and that the activity of the latter is masked by being coupled with antibody.

Ishikawa[40, 51] suggested that the complimentary action of the surface radicals played a role in early amphibian development. Moreover, postulating from Weiss' conception of "molecular ecology", the inducing ability of inductor and the reacting activity of the presumptive epidermis might be inhibited by the coupling of antigen with antibody. The neural inducing agent of the liver protein would be carried by being coupled with the antiserum and the inducing effect would then be generally depressed by the action of surface radicals in the antibody. However, as the specificity of antibodies would differ according to the immunizing method, the induction of mesodermalization might occur in some process of immunization with the liver protein. However, it is not possible to state a definite mechanism about the action of antiserum or of the coupling of liver protein.

SUMMARY

Guinea pig liver proteins, which had been prepared by isoelectric precipitation after centrifugation at low or high speed, were used as the immunizing antigen in rabbits.

Antisera formed against these antigens were used for precipitin reaction with the low or high speed protein antigen.

These antigens, antisera and antigen-antibody complexes were examined for their induction effects on *Triturus* gastrula.

Antiserum against liver "low speed protein" showed very low frequency and poor differentiation in the induction, and the same inducing tendency was also observed in the case of antiserum against liver "high speed protein".

When the antigens were coupled with antibodies, mesodermal inducing ability appeared only in the antigen-antibody complex with one rabbit antiserum but in others the inducing ability was depressed and the frequency of appearance of induced structure was very low.

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EXPLANATION OF PLATES

Plate 1: Archencephalon and eye induced by the lyophilized liver high speed protein (from Ia).

Plate 2: Two notochords and deuterencephalon induced by the precipitate between liver low speed protein and its antiserum (from IIIa).

Plate 3: Two notochords and deuterencephalon induced by the precipitate between liver low speed protein and its antiserum (from IIIa). The graft located over the notochord of the left side. Two notochords shown in the plate fused together and were surrounded by induced muscle in other sections.

Plate 4: Notochord and deuterencephalon induced by the precipitate between liver low speed protein and its antiserum (from IIIa). Two ears were observed on the left side of notochord in other sections.

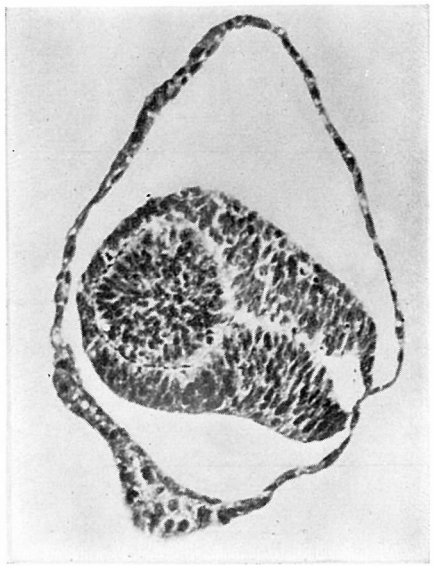

Plate 1

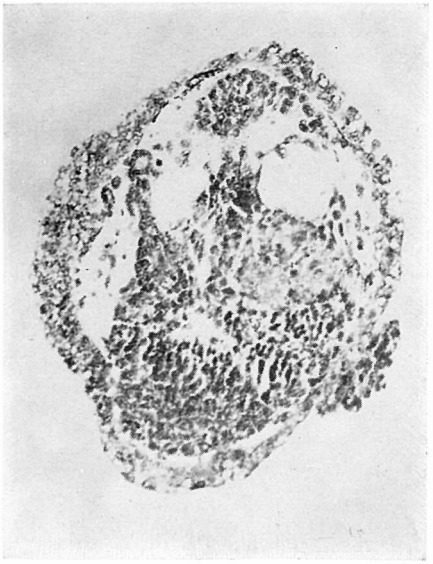

Plate 2

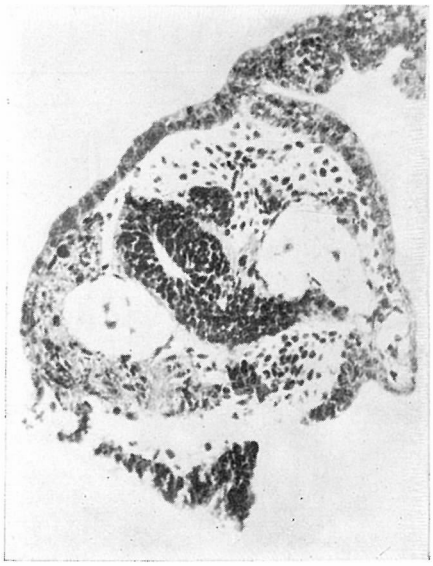

Plate 3

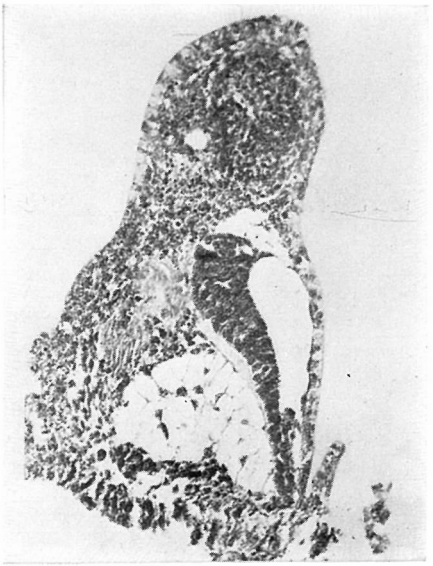

Plate 4