

TUBERCULIN TYPE OF HYPERSENSITIZATION TO CRYSTALLINE VITAMIN B-12 (CYANOCOBALAMIN) INDUCED IN GUINEA PIGS

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EXPERIMENTALLY induced sensitization to central nervous system tissue can cause extensive changes in the CNS of guinea pigs and is referred to as experimental allergic encephalomyelitis. The precise dynamics of the events is not elucidated.¹ One possibility is that certain normal metabolic pathways are specifically interrupted which leads to the CNS changes observed.

The subacute combined degeneration of human spinal cord associated with improper vitamin B₁₂ metabolism has no experimental counterpart. An effort is described of an attempt to interfere with normal vitamin B₁₂ metabolism in the guinea pig by inducing sensitization to vitamin B₁₂. No CNS changes were observed but, in the course of the study, a tuberculin-type cutaneous sensitization to crystalline vitamin B₁₂ was developed. Crystalline vitamin B₁₂ purified from *Streptomyces* fermentation† (as ordinarily used in human therapy) and crystalline vitamin B₁₂ prepared from liver‡ were used.

There have been several reports of hypersensitivity to vitamin B₁₂ in human subjects injected either with *crude* extracts or concentrates prepared from liver or fermentation of *Streptomyces*. In 1950 Young and associates² reported a case of anaphylactic hypersensitivity in a person sensitive to purified liver extracts following the administration of vitamin B₁₂ concentrate made from streptomycin broth. This individual did not react to crystalline vitamin B₁₂.

Bedford³ reported that 14 of 100 individuals, skin tested with concentrates made from *Streptomyces* fermentation liquors, showed positive skin reaction and that 6 showed side effects after intramuscular injection. When these individuals were tested with crystalline material prepared from *Streptomyces* fermentation only 2 showed positive skin tests and none had side effects following intramuscular administration. None of the individuals showed sensitivity

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to purified vitamin B₁₂ prepared from liver. Those individuals who had received large amounts of antibiotics in the past were sensitive to vitamin B₁₂ prepared from *Streptomyces*. Bedford, therefore, attributed the sensitization to by-products rather than to vitamin B₁₂ itself.

Rostenberg and Perkins⁴ demonstrated a tuberculin-type skin sensitization to crystalline vitamin B₁₂ in an individual who was sensitive to cobalt and nickel. Ungley⁵ reported the occurrence of either the immediate type or atopic type of sensitization in 3 individuals to crystalline vitamin B₁₂, the source of which was not given. The fact that the sensitization was transferred to a nonsensitive normal subject, in what appeared to be a Prausnitz-Küstner transfer by serum, rules out the delayed type of sensitization. He stated that in the following 4 years with the use of crystalline vitamin B₁₂ no other cases were encountered. Traina⁶ reported the inability (in a very limited attempt) to produce anaphylactic shock in guinea pigs injected subcutaneously with many very small doses of crystalline vitamin B₁₂. Traina also mentions the anti-anaphylactic effect of vitamin B₁₂.

MATERIALS AND METHODS

Hartley strain guinea pigs of both sexes, weighing from 500 to 650 grams, were used throughout the experiments. They were fed Purina guinea pig chow and alfalfa hay, and were given water ad libitum.

In the first experiment, crystalline vitamin B₁₂ prepared from *Streptomyces* fermentation was used for the initial injection as follows: a solution of the crystals in sterile distilled water or 0.85 per cent sodium chloride solution was emulsified with complete Freund's adjuvants as described previously.⁷ The emulsion for the initial injection contained aqueous vitamin B₁₂, killed dried *Mycobacterium tuberculosis*, Arlacel A, and light mineral oil. Each animal was injected intracutaneously at seven sites, six on the back with 0.05 ml. of emulsion and 0.1 ml. on the ventral aspect of the neck. Each animal received a total of 2.0 mg. of crystalline vitamin B₁₂ and 0.8 mg. of killed tubercle bacilli in a total volume of 0.4 ml. of emulsion. Two control groups of animals were as follows: a group of 8 normal uninjected animals and a group of 8 animals injected with complete Freund's adjuvants without vitamin B₁₂.

Animals were weighed and observed for a period of 28 days after immunization. At this time all animals were skin tested by the intracutaneous injection of an aqueous (distilled water) solution of crystalline vitamin B₁₂ in the right flank. Three different concentrations of crystalline vitamin B₁₂ were used; 200 gammas, 20 gammas and 2 gammas each, in a volume of 0.1 ml. The skin test sites were observed for a period of 72 hours; measurements were made of the erythema as well as of the type and degree of swelling after 5, 24, 48, and 72 hours.

The second experiment included two groups of 8 animals each. One group received crystalline vitamin B₁₂ prepared from *Streptomyces* fermentation in complete Freund's adjuvants as outlined above. The second group received only complete Freund's adjuvant and served as controls. At 13 days all 16

animals were skin tested on the right side with 100 gammas of the vitamin B₁₂ prepared from Streptomyces. At 20 days and at 27 days these animals also received on the same side (right) two skin test doses of 100 gammas and 10 gammas of vitamin B₁₂. The skin test sites were examined and measured as in the first experiment. Three of the experimental animals which showed the highest degree of sensitization as judged by skin test reaction at 27 days were skin tested on the previously uninjected side (left) with 40 gammas of crystalline vitamin B₁₂ derived from *liver* in a volume of 0.2 ml. Three animals injected with Freund's complete adjuvants alone were similarly skin tested at the same time.

The remaining 5 animals in each group were skin tested at 40 days with 20 gammas of crystalline vitamin B₁₂ from liver in a volume of 0.1 ml. These skin tests were done on the previously untested left side.

RESULTS

The results are summarized in Tables I and II. Table I shows that all animals injected originally with crystalline vitamin B₁₂ (Streptomyces) in Freund's complete adjuvants developed skin sensitization to 200 gammas of crystalline vitamin B₁₂ injected intracutaneously. Only 2 of the 8 animals showed positive tests to 20 gammas of vitamin B₁₂ and none of these 8 animals reacted to 2 gammas of vitamin B₁₂. No reaction was observed after 5 hours. Skin reactions reached their peak between 24 and 48 hours and were still visible in most instances 72 hours after injection. This indicated a tuberculin or delayed type of hypersensitivity. All 8 of the control animals injected with com-

TABLE I. SKIN REACTIONS IN 8 GUINEA PIGS SENSITIZED AND SKIN TESTED WITH CRYSTALLINE VITAMIN B₁₂ (CYANOCOBALAMIN) DERIVED FROM STREPTOMYCES FERMENTATION*

AMOUNT†	TIME OF READING OF REACTION (HOURS AFTER INJECTION OF TEST DOSE)			
	5	24	48	72
200	Neg.	WDdP-Ind. 8‡	WDP-Ind. 7	WDP-Ind. 8
20	Neg.	WDP-Ind. 6	IDP-Ind. 7	WDP-Ind. 7
200	Neg.	WDR-Ind. 10	WDdP-Ind. 10	WDP-Ind. 10
20	Neg.	WDP-Ind. 5 × 4	P-Nod. 4	P Nod. 3
200	Neg.	WDR-Ind. 10 × 8	WDP-Ind. 10 × 7	ID-P-Ind. 8
20	Neg.	Neg.	Neg.	Neg.
200	Neg.	WDR-Ind. 9	WDdP-Ind. 9 × 6	IDP-Ind. 7
20	Neg.	Neg.	Neg.	Neg.
200	Neg.	WDdP-Ind. 9 × 6	WDdP-Ind. 7	IDP-Ind. 8 × 5
20	Neg.	Neg.	Neg.	Neg.
200	Neg.	WDP-Ind. 8	WDR-Ind. 10	IDP-Ind. 7
20	Neg.	Neg.	Neg.	Neg.
200	Neg.	IDdP-Ind. 7	IDP-Ind. 5	IDP-Nod. 5
20	Neg.	Neg.	Neg.	Neg.
200	Neg.	WDP-Ind. 7 × 5	IDdP-Ind. 7 × 5	IDP-Nod. 5
20	Neg.	Neg.	Neg.	Neg.

Legend: WD—Well defined; ID—ill defined; Nod.—nodule; Ind.—induration (thickness 1 to 3 mm.); P—pink; R—red; dP—dark pink.

*Controls injected with adjuvants alone or uninjected (8 per group) were negative with occasional ill-defined nodules 2 to 3 mm. in diameter.

†Test dose (gammas) in 0.1 ml. volume.

‡Dimensions are in millimeters and one number is diameter. Two numbers are 2 dimensions if reaction was irregular.

plete Freund's adjuvants and the 8 uninjected controls showed no skin reaction to the injection of 200 gammas of crystalline vitamin B₁₂.

Table II shows the results of using crystalline vitamin B₁₂ prepared from Streptomyces fermentation for the initial injection and skin tests and that derived from liver for final skin tests. Not presented in the table are the skin test reactions observed at 13 days and at 20 days following the intracutaneous injection of crystalline vitamin B₁₂ prepared by Streptomyces fermentation. The group injected with crystalline vitamin B₁₂ combined with Freund's adjuvants showed weak but positive skin reactions when tested at 13 days and stronger reactions at 20 days. Skin tests done at 27 days with Streptomyces crystalline vitamin B₁₂ are presented in the table. These reactions ranged from well-defined pink and indurated zones measuring 14 mm. in diameter to a negative reaction seen in one animal which was tested with 100 gammas of crystalline vitamin B₁₂. Four of the 8 animals showed sensitization at the 10-gamma skin site. The animals treated at 35 days with vitamin B₁₂ derived from liver and injected with 40 gammas in a 0.2 ml. volume were highly positive. All of the remaining 5 animals skin tested at 40 days showed reactions to 20 gammas in 0.1 ml. of crystalline liver vitamin B₁₂.

TABLE II. SKIN REACTIONS IN GUINEA PIGS SENSITIZED WITH CRYSTALLINE VITAMIN B₁₂ (STREPTOMYCES) IN COMPLETE FREUND ADJUVANTS AND SKIN TESTED WITH CRYSTALLINE VITAMIN B₁₂ DERIVED FROM STREPTOMYCES AND FROM LIVER*

TEST DOSE SOURCE† AMOUNT	TEST DAY	TIME OF READING OF REACTION (HOURS AFTER INJECTION OF TEST DOSE)		
		24	48	72
Strep. 100	27	WDP-Ind. 14‡	WDP-Ind. 14	WDP-Ind. 14
Strep. 10	27	P-Ind. 9	WDP-Ind. 12	WDP-Ind. 12
Liver 40	35	WDdP-Ind. 16	WDdP-Ind. 16	WDdP-Ind. 14
Strep. 100	27	P-Ind. 14 × 10	WDP-Ind. 11	WDP-Ind. 15
Strep. 10	27	P-Ind. 6	Neg.	Neg.
Liver 40	35	WDP-Ind. 11	WDP-Ind. 12	WDP-Ind. 12
Strep. 100	27	WDP-Ind. 12	WDP-Ind. 12	WDP-Ind. 12
Strep. 10	27	WDP-Ind. 12	WDP-Ind. 12	WDP-Ind. 12
Liver 40	35	WDP-Ind. 15	WDP-Ind. 11	WDP-Ind. 9
Strep. 100	27	P-Ind. 10	P-SS-10	P-SS 10
Strep. 10	27	Neg.	Neg.	Neg.
Liver 20	40	WDP-Ind. 13	WDP-Ind. 10	P-Ind. 10
Strep. 100	27	P-S 12 × 8	P-S 9	Neg.
Strep. 10	27	P-Ind. 13 × 10	P-Ind. 10	Neg.
Liver 20	40	P-Ind. 11	P-Ind. 8	P-Ind. 9
Strep. 100	27	Neg.	Neg.	Neg.
Strep. 10	27	Neg.	Neg.	Neg.
Liver 20	40	WDP-Ind. 12	P-Ind. 8	P-Ind. 8
Strep. 100	27	WDP-Ind. 14	WDdP-Ind. 12	WDP-Ind. 12
Strep. 10	27	P-Nod. 5	P-Nod. 4	Neg.
Liver 20	40	P-Ind. 11	WDP-Ind. 9	P-Nod. 6
Strep. 100	27	P-Ind. 11 × 8	P-Ind. 6	Neg.
Strep. 10	27	Neg.	Neg.	Neg.
Liver 20	40	WDP-Ind. 14 × 11	IDP-Ind. 9	P-Nod. 6

Legend: See Table I. SS—soft swelling.

*8 controls injected with complete Freund adjuvants less vitamin B₁₂ were negative.

†Strep.—Vitamin B₁₂ derived from Streptomyces. Liver—Vitamin B₁₂ derived from liver.

‡See Table I.

No reactions were visible at 5 hours following the injection of the skin test doses of crystalline vitamin B₁₂ derived from *Streptomyces* or from that derived from liver. The reactions reached their peak in 24 to 48 hours indicating a delayed type of hypersensitivity to the vitamin B₁₂.

The control animals which received complete Freund's adjuvants alone showed negative skin tests, although all of these animals had received the same series of skin test injections of aqueous vitamin B₁₂ as the experimental animals.

DISCUSSION

The original purpose of our experiments, i.e., to produce a disease in guinea pigs similar to subacute degeneration of the cord in man, was not realized. This may have been due to an inadequate immunization or sensitization and the relative resistance of the guinea pig to vitamin B₁₂ deficiency. However, in the course of these experiments we demonstrated that the guinea pigs could be sensitized to a vitamin which is essential for the growth and maintenance of most mammals. The sensitization developed in these experiments was to crystalline vitamin B₁₂ itself rather than to contaminants which might be present in its preparation. This was demonstrated by the use of crystalline vitamin B₁₂ prepared from *Streptomyces* fermentation for sensitization with cross skin test reactions to vitamin B₁₂ prepared from liver. Due to the difficulty of obtaining crystalline vitamin B₁₂ prepared from liver, this material could only be used for skin testing after the sensitization was produced by the use of crystalline vitamin B₁₂ prepared from *Streptomyces*.

These experiments were carried out by the use of complete Freund's adjuvants and no effort was made to produce sensitization by any other means. In the course of the experiments, however, the control animals were skin tested several times and did not develop sensitization although skin testing was favorable for the production of delayed hypersensitization.

The sensitization was demonstrated to be of the delayed or tuberculin type by temporal sequence of the skin test reaction. No attempt was made to transfer this sensitization to other animals by cells. Unreported here was the unsuccessful attempt to demonstrate neutralizing antibodies to vitamin B₁₂ by the use of serum from a highly sensitive animal in a test for vitamin B₁₂. The inability to produce pathological changes may be due to a lack of circulating antibody. The ability of cells of an animal sensitized with a delayed type of sensitization to neutralize a pharmacological substance, such as crystalline vitamin B₁₂ or any other physiologically active material, had not been demonstrated.

The significance of sensitization of guinea pigs to crystalline vitamin B₁₂ in regard to the clinical application of vitamin B₁₂ in humans is unknown. It only points to the possibility that one can sensitize to crystalline vitamin by artificial means. It should be kept in mind that sensitization is possible and therefore that the best method of injection should be one which does not favor sensitization.

SUMMARY

Crystalline vitamin B₁₂ (cyanocobalamin), emulsified with Freund's adjuvants including killed tubercle bacilli and injected intracutaneously, is capable

of sensitizing guinea pigs to crystalline vitamin B₁₂. The specificity of sensitization to vitamin B₁₂ was ascertained by sensitizing with cyanocobalamin derived from *Streptomyces* fermentation liquor and by a skin test with crystallin vitamin B₁₂ derived from liver. The sensitization induced is of the tuberculin type and reaches its peak in 24 to 48 hours after the skin test injection.

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