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ATTEMPTS TO INDUCE SENSITIZATION IN GUINEA PIGS WITH NICKEL COMPLEXES

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Abstract. A technique for the induction of sensitization in pigs with a nickel–alanine conjugate has been reported by other investigators. Similar results were observed in our experiments in mini-pigs. However, using the identical technique, we were unable to induce sensitization in guinea pigs with $NiSO_4$, with nickel–alanine or other nickel–amino acid complexes, or with a complex of nickel with soluble guinea pig skin extract. These results indicate that nickel–amino acid complexes and nickel–guinea pig skin complex were not antigenic in guinea pigs with this technique. Hypotheses for the apparent failure to induce sensitization are proposed.

Key words: Nickel sensitization; Nickel complexes; Guinea pig sensitization; Nickel complexes in guinea pigs and mini-pigs; Induction of nickel sensitization

Experimental sensitization to nickel in guinea pigs has been reported by some investigators (10, 13, 14). Their results, however, have not been confirmed by other investigators (4, 5, 12). Nilzén & Wikström (10) reported a method for sensitizing animals to nickel by repeated topical applications of aqueous nickel sulfate solutions containing sodium lauryl sulfate. Samitz & Pomerantz (12) were unable to demonstrate sensitization when employing this technique; their results showed that sodium lauryl sulfate, in combination with nickel sulfate, produces a local irritation rather than allergic reactions. Utilizing their guinea pig maximization test, Magnusson & Kligman (9) were able to sensitize guinea pigs to nickel, though their results were not consistent in every animal in which induction was attempted. Jansen et al. (6), on the other hand, reported consistent induction of sensitization in four pigs with a nickel–alanine conjugate. In their experiments, challenge tests with the nickel complex showed weak reactions; simultaneous tests with nickel sulfate, however, produced strongly positive, delayed reactions.

We reported a technique for consistent induction of high levels of sensitization to chromium salts; however, attempts to sensitize guinea pigs with nickel sulfate using our experimental model were equivocal (3). The technique used by Jansen et al. (6) in mini-pigs was attractive because of its simplicity and the time factors involved. We therefore thought it advisable to test the efficacy of nickel sulfate and various nickel complexes to determine if this technique was suited to the induction of delayed hypersensitivity to nickel in small laboratory animals (guinea pigs).

The complexes used for induction were *dl*-Ni–alanine ($Ni(ALA)_2$), Ni–tyrosine ($Ni(TYR)_2$), Ni–glycine ($Ni(GLY)_2$), Ni–β-alanine ($Ni(\beta\text{-}ALA)_2$), Ni–phenylalanine ($Ni(\phi\text{-}ALA)_2$), and nickel–guinea pig skin extract (Ni–GPS). The guinea pig skin–hapten complex was selected on the basis of experiments by Salvin & Smith (11) and Chase & Kawata (2).

Challenge testing was carried out with nickel sulfate, the nickel–amino acid complexes, nickel–guinea pig skin complex, alanine, and guinea pig skin extract.

MATERIAL AND METHODS

Preparation of $NiSO_4$ and Nickel Complexes. Sensitizers and Elicitors

Preparation of $NiSO_4$

The sensitizing solution of $NiSO_4$ was prepared by dissolving 0.8441 g of $NiSO_4 \cdot 6H_2O$ in pss and diluting to 100 ml with physiological saline solution (pss). This represented a 3.21×10^{-2} M solution. Sterilization was by Millipore filtration.

The eliciting solution was prepared by diluting 1 ml of the sensitizing solution to 10 ml with sterile pss.

Preparation of Alanine

An alanine solution was prepared by dissolving 0.2799 g *dl*-alanine in pss and diluting to 100 ml with pss. This solution was 3.14×10^{-2} M. Sterilization was by Millipore filtration. The eliciting solution of ALA was prepared by diluting 2 ml of the 3.14×10^{-2} M solution to 10 ml with sterile pss. The resulting solution was 6.28×10^{-3} M.

Preparation of $Ni(ALA)_2$

One hundred ml of distilled water containing 1 g of *dl*-alanine was heated nearly to boiling and treated with 1 g of $NiCO_3$ added in five portions with vigorous stirring. The system was boiled for a few minutes and then filtered while still hot through Whatman no. 1 paper. The filtrate was stored in a vacuum desiccator for 2 days to reduce its volume to about 25 ml. The filtrate was then treated with 100 ml of ethanol to precipitate the $Ni(ALA)_2 \cdot 4H_2O$. The pale blue crystals were filtered through Whatman no. 1 paper on a Buchner funnel, washed once with 20 ml of ethanol and air dried. The recovered product was stored in a vacuum desiccator. Yield was 1.4 g.

The sensitizing solution of $Ni(ALA)_2$ was prepared by dissolving 1 g of the $Ni(ALA)_2 \cdot 4H_2O$ in pss and diluting to 100 ml with pss. This solution was equivalent to 3.25×10^{-2} M. The Ni $(ALA)_2$ solution was sterilized by passage through a 0.45 μm Millipore filter into a sterile serum bottle.

The eliciting solution of $Ni(ALA)_2$ was prepared by diluting 1 ml of the sensitizing solution (3.25×10^{-2} M) to 10 ml with sterile pass in a sterile serum bottle.

Preparation of $Ni(TYR)_2$

One g of *dl*-tyrosine and 2 g of $NiCO_3$ were heated to boiling in 100 ml of distilled water. The hot system was immediately filtered. The residue was washed three times with 10 ml portions of boiling water, and the washings were added to the filtrate. The filtrate was transferred to a 250 ml beaker, cooled, and stored in a vacuum desiccator. Two days later, the volume was reduced to approximately 50 ml, and the pale blue crystals of $Ni(TYR)_2 \cdot 4H_2O$ were recovered by filtration through Whatman no. 1 paper. The crystals were washed with ethanol, air dried and stored in a vacuum desiccator. Yield was 1 g.

The $Ni(TYR)_2$ sensitizing solution was prepared by dissolving 0.1249 g of Ni $(TYR)_2 \cdot 4H_2O$ in 10 ml of pss. This solution was sterilized by passage through a Millipore filter.

The Ni $(TYR)_2$ eliciting solution was prepared by diluting 1 ml of the sensitizing solution to 10 ml with pss.

Preparation of Ni $(GLY)_2$

One g of glycine was dissolved in 100 ml of distilled water and heated to boiling. One g of $NiCO_3$ was added in several small portions with vigorous stirring, and the system was boiled for 5 min. The hot solution was filtered through Whatman no. 1 paper, and the filtrate was transferred to a 250 ml beaker. The filtrate was allowed to cool and stored in a vacuum desiccator. After 5 days in vacuum, when the volume was reduced to approximately 25 ml, 100 ml of ethanol was added. The blue crystals of $Ni(GLY)_2 \cdot 4H_2O$ were recovered by filtration, washed with 10 ml of ethanol and air dried. Yield was 1.2 g.

The $Ni(GLY)_2$ sensitizing solution was prepared by dissolving 0.0832 g of $Ni(GLY)_2 \cdot 4H_2O$ in 10 ml of pss. This solution was sterilized by Millipore filtration and stored in a sterile serum vial.

The $Ni(GLY)_2$ elicitor solution was prepared by diluting 1 ml of the sensitizing solution to 10 ml with sterile pss.

Preparation of $Ni(\beta\text{-}ALA)_2$

One gram of β-alanine was dissolved in 100 ml of distilled water and heated to boling. One gram of $NiCO_3$ was added in several small portions with vigorous stirring, and the system was boiled for 5 min. The hot system was filtered through Whatman no. 1 paper, and the filtrate was transferred to a 250 ml beaker. The beaker was allowed to cool and then stored in a vacuum desiccator for 5 days. After this time, the volume was reduced to approximately 50 ml. One hundred ml of alcohol was added to precipitate the $Ni(\beta\text{-}ALA)_2$. The blue crystals were recovered by filtration, washed with alcohol and stored in a vacuum desiccator. Yield was 0.4 g.

The $Ni(\beta\text{-}ALA)_2$ sensitizing solution was prepared by dissolving 0.0787 g of $Ni(\beta\text{-}ALA)_2 \cdot 4H_2O$ in 10 ml of pss and passing the resulting solution through a 0.45 μm Millipore filter for sterilization.

The eliciting solution was prepared by diluting 1 ml of sensitizing solution to 10 ml with pss.

Preparation of $Ni(\phi\text{-}ALA)_2$

Five hundred mg of $NiC0_3$ and 500 mg of phenylalanine were weighed into a 100 ml beaker and treated with 50 ml of distilled water. This system was heated to near boiling and then poured into 1 liter of distilled water at room temperature, which was then heated to boiling and boiled for 5 min. The hot system was filtered through Whatman no. 1 paper and the filtrate was allowed to stand (covered) for 5 days. After this time, the pale blue crystals of Ni $(\phi\text{-}ALA)_2 \cdot 4H_2O$ were recovered by filtration, washed with alcohol and dried in a vacuum desiccator. Yield was 0.3 g.

The $Ni(\phi\text{-}ALA)_2$ sensitizing solution was prepared by dissolving 0.1208 g of $Ni(\phi\text{-}ALA)_2 \cdot 4H_2O$ in 10 ml of pss. Sterilization was by Millipore filtration.

The $Ni(\phi\text{-}ALA)_2$ eliciting solution was prepared by diluting 1 ml of the sensitizing solution to 10 ml with sterile pss.

Preparation of Ni–GPS and GPS

Shaved skin trimmed of fat from the backs and sides of two sacrificed guinea pigs was cut into pieces approximately 1×1 cm and stored in pss under refrigeration. The minced guinea pig skin was homogenized in a Waring blendor in twenty separate, 1 g portions each with 10 ml pss. The homogenizates were pooled and filtered through coarse paper. The filtrate was centrifuged, and the supernatant liquid was transferred to a 250 ml beaker. The solution was stored in a vacuum desiccator under refrigeration for 7 days until its volume was reduced to approximately 50 ml. The contents of the beaker were treated with 100 ml of acetone and allowed to stand overnight in the cold. The precipitated protein was filtered through Whatman no. 1 paper on a Buchner funnel and dried in a vacuum desiccator. Yield was 220 mg.

Two hundred mg of the soluble guinea pig skin protein was dissoved in 100 ml pss. Fifty ml of this solution was treated with 50 ml of pss. and 50 ml of the protein solution was treated with 50 ml of 1.00×10^{-2} M $NiCl_2$ in pss (0.2377

Table I. *Sensitization attempted with 3×10^{-2} M $NiSO_4$*

Elicitors	Reactions 0	+/−	1+	2+
3×10^{-3} M $NiSO_4$	20/34	11/34	3/34	0/34
3×10^{-3} M $Ni(ALA)_2$	25/34	4/34	5/34	0/34
6×10^{-3} M ALA	18/18	0/18	0/18	0/18
pss	6/6	0/6	0/6	0/6
1:10 Ni-GPS	17/18	1/18	0/18	0/18
1:10 GPS	17/18	1/18	0/18	0/18
3×10^{-3} M $Ni(TYR)_2$	4/4	0/4	0/4	0/4
3×10^{-3} M $Ni(\phi\text{-ALA})_2$	4/4	0/4	0/4	0/4
3×10^{-3} M $Ni(\beta\text{-ALA})_2$	8/12	4/12	0/13	0/12
3×10^{-3} M $Ni(GLY)_2$	10/12	2/12	0/12	0/12

g $NiCl_2 \cdot 6H_2O$ dissolved in and diluted to 100 ml with pss). Both solutions were incubated at 0°C for 48 hours.

After the incubation, both the soluble guinea pig skin protein (GPS) solution and the soluble guinea pig skin protein treated with the nickel chloride (Ni-GPS) solution were subjected to dialysis against six 300 ml changes of pss over a 12 day period at 0°C. The solutions within the dialysis tubing were then centrifuged, and the supernatant liquids were removed and diluted to 100 ml with pss. Both the Ni-GPS and the GPS were sterilized by Millipore filtration and stored in sterile serum bottles. These solutions were used as sensitizing solutions, and 1:10 dilutions of these solutions with sterile pss were used as elicitors.

Animal Experiments

We followed exactly the sensitization and challenge procedures reported by Berrens (1) and Jansen et al. (6): timing and dosage were identical.

Attempts were made to sensitize male, Hartley strain guinea pigs (300 g). The procedure is as follows:

Day 1: administer 0.2 ml of sensitizer solution by intracutaneous injection in the hairless area behind the right ear.

Day 3: administer 0.4 ml of sensitizer solution by intracutaneous injection in the hairless area behind the left ear.

Day 6: administer 0.4 ml of the sensitizer solution by intracutaneous injection into the hairless area behind the right ear.

On the 12th day after the initial sensitizing injection, the sides of the animals were shaved with an electric clipper, and the animals were tested by intradermal injection of 0.1 ml of the elicitor solution. Reactions to these injections were read after 48 hours using the following notations:

0	no detectable reaction
+/−	equivocal reaction
1+	distinct redness with underlying papule
2+	large (>5 mm) erythema and infiltrated papule

We also repeated Jansen et al.'s experiments using the hybrid-cross between the Hampshire and the Checkered White species of pig (16 kg). Six pigs were each injected with 0.2 ml of 1% $Ni(ALA)_2 \cdot 4H_2O$ on the first day and then with 0.4 ml of the same solution on the fourth and seventh days. Four pigs served as controls. On the thirteenth day after the initial injection, all 10 animals were challenged by intradermal injections with 0.1 ml of 0.1% of $Ni(ALA)_2 \cdot 4H_2O$. 0.1 ml of 0.1% $NiSO_4 \cdot 6H_2O$ and 0.1 ml of pss. Reactions were read after 24 hours and after 48 hours.

RESULTS

Results of skin testing are shown in Tables I–VIII.

They are also compared with those reported by Jansen et al. (7) in Table IX.

DISCUSSION

Berrens (1) advanced the concept that hapten–amino acid conjugates may be valuable tools for the successful sensitization of experimental animals to contact allergens. This hypothesis was substantiated by experimental work with nickel in pigs. Jansen et al. showed that, comparatively, nickel-alanine complex was a better sensitizer than $NiSO_4$ per molecule of applied substance (6). In these experiments, they reported positive reactions at 24 hours and their reversion to negative at 48 hours. We were able to confirm these results in our experiments in mini-pigs. Can we call such reactions definitive evidence of sensitization? Are such reaction patterns typical for the pig? The 24 hour reaction certainly did not represent an irritant phenomenon, as the control animals in these experiments showed no reactions. Can we consider these reactions as abortive delayed hypersensitivity or weak antibody mediated allergic reactions? Is the amino acid–hapten conjugate a potent sensitizer? Is the failure due to the technique of sensitization or to the amino acid carrier?

Using the identical technique, we used guinea pigs

Table II. *Sensitization attempted with 3×10^{-2} M $Ni(ALA)_2$*

Elicitors	Reactions 0	+/−	1+	2+
3×10^{-3} M $NiSO_4$	23/48	8/48	16/48	1/48
3×10^{-3} M $Ni(ALA)_2$	35/48	9/48	4/48	0/48
6×10^{-3} M ALA	22/22	0/22	0/22	0/22
pss	6/6	0/6	0/6	0/6
1:10 Ni-GPS	18/22	4/22	0/22	0/22
1:10 GPS	20/22	2/22	0/22	0/22
3×10^{-3} M $Ni(TYR)_2$	4/4	0/4	0/4	0/4
3×10^{-3} M $Ni(\phi\text{-ALA})_2$	4/4	0/4	0/4	0/4
3×10^{-3} M $Ni(\beta\text{-ALA})_2$	9/12	2/12	1/12	0/12
3×10^{-3} M $Ni(GLY)_2$	10/12	2/12	0/12	0/12

Table III. *Sensitization attempted with 3×10^{-2} M $Ni(TYR)_2$*

Elicitors	Reactions 0	+/−	1+	2+
3×10^{-3} M $NiSO_4$	3/4	0/4	1/14	0/4
3×10^{-3} M $Ni(ALA)_2$	3/4	1/4	0/4	0/4
6×10^{-3} M ALA				
pss				
1:10 Ni-GPS				
1:10 GPS				
3×10^{-3} M $Ni(TYR)_2$	4/4	0/4	0/4	0/4
3×10^{-3} M $Ni(\phi\text{-ALA})_2$	4/4	0/4	0/4	0/4
3×10^{-3} M $Ni(\beta\text{-ALA})_2$				
3×10^{-3} M $Ni(GLY)_2$				

Table IV. *Sensitization attempted with 3×10^{-2} M $Ni(GLY)_2$*

Elicitors	Reactions 0	+/−	1+	2+
3×10^{-3} M $NiSO_4$	9/12	3/12	0/12	0/12
3×10^{-3} M $Ni(ALA)_2$	10/12	2/12	0/12	0/12
6×10^{-3} M ALA				
pss	6/6	0/6	0/6	0/6
1:10 Ni-GPS				
1:10 GPS				
3×10^{-3} M $Ni(TYR)_2$				
3×10^{-3} M $Ni(\phi\text{-ALA})_2$				
3×10^{-3} M $Ni(\beta\text{-ALA})_2$	10/12	1/12	1/12	0/12
3×10^{-3} M $Ni(GLY)_2$	11/12	1/12	0/12	0/12

Table V. *Sensitization attempted with 3×10^{-2} M $Ni(\beta\text{-}ALA)_2$*

Elicitors	Reactions 0	+/−	1+	2+
3×10^{-3} M $NiSO_4$	9/12	1/12	1/12	1/12
3×10^{-3} M $Ni(ALA)_2$	9/12	2/12	1/12	0/12
6×10^{-3} M ALA				
pss	6/6	0/6	0/6	0/6
1:10 Ni-GPS				
1:10 GPS				
3×10^{-3} M $Ni(TYR)_2$				
3×10^{-3} M $Ni(\phi\text{-ALA})_2$				
3×10^{-3} M $Ni(\beta\text{-ALA})_2$	11/12	1/12	0/12	0/12
3×10^{-3} M $Ni(GLY)_2$	9/12	1/12	2/12	0/12

Table VI. *Sensitization attempted with 3×10^{-2} M $Ni(\phi\text{-}ALA)_2$*

Elicitors	Reactions 0	+/−	1+	2+
3×10^{-3} M $NiSO_4$	2/4	2/4	0/4	0/4
3×10^{-3} M $Ni(ALA)_2$	4/4	0/4	0/4	0/4
6×10^{-3} M ALA				
pss				
1:10 Ni-GPS				
1:10 GPS				
3×10^{-3} M $Ni(TYR)_2$	4/4	0/4	0/4	0/4
3×10^{-3} M $Ni(\phi\text{-ALA})_2$	3/4	1/4	0/4	0/4
3×10^{-3} M $Ni(\beta\text{-ALA})_2$				
3×10^{-3} M $Ni(GLY)_2$				

Table VII. *Sensitization attempted with Ni-GPS*

Elicitors	Reactions 0	+/−	1+	2+
3×10^{-3} M $NiSO_4$	5/18	4/18	7/18	1/18
3×10^{-3} M $Ni(ALA)_2$	13/18	3/18	2/18	0/18
6×10^{-3} M ALA	18/18	0/18	0/18	0/18
pss				
1:10 Ni-GPS	18/18	0/18	0/18	0/18
1:10 GPS	17/18	1/18	0/18	0/18
3×10^{-3} M $Ni(TYR)_2$				
3×10^{-3} M $Ni(\phi\text{-ALA})_2$				
3×10^{-3} M $Ni(\beta\text{-ALA})_2$				
3×10^{-3} M $Ni(GLY)_2$				

Table VIII. *Control animals*

Elicitor	Reactions 0	+/−	1+	2+
3×10^{-3} M $NiSO_4$	19/33	3/33	11/33	0/33
3×10^{-3} M $Ni(ALA)_2$	23/33	4/33	5/33	1/33
6×10^{-3} M ALA	12/12	0/12	0/12	0/12
pss	6/6	0/6	0/6	0/6
1:10 Ni-GPS	12/12	0/12	0/12	0/12
1:10 GPS	12/12	0/12	0/12	0/12
3×10^{-3} M $Ni(TYR)_2$	3/3	0/3	0/3	0/3
3×10^{-3} M $Ni(\phi\text{-ALA})_2$	3/3	0/3	0/3	0/3
3×10^{-3} M $Ni(\beta\text{-ALA})_2$	9/12	2/12	1/12	0/12
3×10^{-3} M $Ni(GLY)_2$	8/12	4/12	0/12	0/12

Table IX. *Sensitization with 1% $Ni(ALA)_2 \cdot 4H_2O$ in pigs*

	Elicitors			
	0.1% $NiSO_4 \cdot 6H_2O$ Reactions after		0.1% $Ni(ALA)_2 \cdot 4H_2O$ Reactions after	
	24 hrs	48 hrs	24 hrs	48 hrs
Results of Jansen et al. (7) in animals sensitized with 1% $Ni(ALA)_2 \cdot 4H_2O$	1 cm	2.5 mm	Neg	Neg
	1 cm	Neg	5 mm	Neg
	1 cm	Neg	5 mm	Neg
	15 mm	1 cm	1 cm	Neg
Jansen et al.'s control	Neg	Neg	Neg	Neg
	Neg	Neg	Neg	Neg
	Neg	Neg	Neg	Neg
	Neg	Neg	Neg	Neg
Our results in animals sensitized with 1% $Ni(ALA)_2 \cdot 4H_2O$	6 mm	Neg	6 mm	Neg
	6 mm	Neg	Neg	Neg
	6 mm	Neg	Neg	Neg
	6 mm	Neg	Neg	Neg
	6 mm	Neg	Neg	Neg
	4 mm	Neg	6 mm	Neg
Our controls	Neg	Neg	Neg	Neg
	Neg	Neg	Neg	Neg
	Neg	Neg	Neg	Neg
	Neg	Neg	Neg	Neg

to test $NiSO_4$, various Ni–amino acid complexes and a nickel complex with soluble guinea pig skin protein extract.

We were unable to induce sensitization in guinea pigs with $NiSO_4$ with the Jansen et al. technique. Further, we were unable to induce sensitization in guinea pigs with nickel–alanine complex with this method. Although 16/48 (33%) guinea pigs had +1 reactions with $NiSO_4$ as an elicitor, 11/33 (33%) control animals showed similar reactions.

Other nickel–amino acid complexes (Ni–tyrosine, Ni–glycine, Ni–β-alanine and Ni–phenylalanine) were ineffective as sensitizers.

Guinea pigs treated with nickel–guinea pig skin complex showed +1 reactions in 7/18 (39%) test animals and 11/33 (33%) control animals with $NiSO_4$ as an elicitor.

If we are to assume that sensitization can be induced in pigs with this technique, is it possible that the identical concentration and dose schedule, applicable to pigs, is not relevant to guinea pigs. Could this be explained by species dependence: do guinea pigs have a natural tolerance to nickel? Is there a differential ability in the pig and the guinea pig to react with allergic contact sensitivity to nickel? Does the antigenicity of these conjugates differ in the two species? Or is it possible that a state of immunologic unresponsiveness could have been achieved with what could have been a high concentration of nickel for the guinea pig but correct for the mini-pig. The dosage of $Ni(ALA)_2 \cdot 4H_2O$ in the guinea pig corresponded to 33 mg/kg, while the dosage in the mini-pig corresponded to only 0.625 mg/kg. High doses of hapten may destroy or inhibit antigen-sensitive cells as well as their precursors, thereby impairing the production of sensitized lymphocytes. The ineffectiveness of $NiSO_4$ and $Ni(ALA)_2$ with the Jansen et al. technique also could be explained by the theory that the escape of allergic chemicals from local sites into the blood evokes a measure of unresponsiveness (8). Incorporation of $NiSO_4$, $Ni(ALA)_2$ or Ni–GPS into Freund's complete adjuvant, however, did not produce sensitization in guinea pigs (unpublished study).

Chase & Kawata (2), on the other hand, used guinea pig epithelium as the carrier for picryl chloride in sensitization experiments and reported that contact-type sensitivity to picryl chloride arose strikingly when compared with the intensity of sensitization achieved with picryl chloride injected intradermally. Our experiments with Ni–guinea pig skin gave inconsistent and low grade reactions (+1 in 7/18 animals). The differences between controls and treated guinea pigs were not significant. Could

this have been the result of using a sensitizer of a quite different class, or to carrier-specific dependence, or that significantly greater quantities of Ni in a rapidly diffusible form may potentiate desensitization rather than sensitization?

In essence: we failed to induce sensitization with $NiSO_4$, nickel–amino acid complexes and nickel–guinea pig skin complex in guinea pigs with the Jansen et al. technique. Hypotheses for these findings are proposed.

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