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**FERRIC NITRILOTRIACETATE (Fe-NTA)-INDUCED “F₁
AMYLOIDOSIS” AND SOME CONSIDERATIONS ABOUT
ITS DEVELOPING MECHANISM**

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Awai *et al.* (1) reported the occurrence of “hemochromatosis” in rats and rabbits after more than 130 daily intraperitoneal injections of ferric nitrilotriacetate (Fe-NTA).

For the purpose of observing the difference in the pathological and histochemical findings between Fe-NTA-induced “hemochromatosis” mice and their progeny as treated likewise an attempt was made to administer Fe-NTA to ICR/JCL mice obtained by reciprocal crossing of both sexes injected with more than 126 injections of the same agent.

Consequently the progeny given 126–502 injections of Fe-NTA were found to reveal severe generalized amyloidosis (abbreviated as “F₁ amyloidosis”) which has not as yet been reported.

It is a subject of great interest to explore the mechanisms whereby “F₁ amyloidosis” has been induced.

Besides, transient immunological unresponsiveness induced with sheep erythrocytes in the offspring of their maternal mice immunized with sheep red blood cells during pregnancy (23) is noted in that the difference of an immune response between maternal mice and their offspring, both of which were given the same antigen for several days, was evidenced.

“F₁ amyloidosis”: One hundred and twenty-three mice used in the present study consisted of 26 parental mice (13 males and 13 females) and 97 F₁ mice (40 males and 57 females) obtained by reciprocal crossing of above parental mice.

Twenty-three ICR/JCL mice of both sexes which were sex-segregated at 4 weeks after birth were at 6 weeks of age injected intraperitoneally with 0.5–1.0 mg (0.014% watery solution) of Dojindo Co. ferric nitrilotriacetate ($\text{C}_6\text{H}_6\text{NO}_6\text{Fe}$, M.W. 244) per 100 g body weight, once a day, 6 times per week. Female mice receiving the above Fe-NTA treatment for approximately 5 months were mated with male mice as treated likewise to obtain their offspring.

Immediately after crossing, both males and females were separately and individually encaged to help expected conception and to avoid excess fighting among males which may occur following crossing.

These parental mice of both sexes were free from the Fe-NTA injection throughout the gestation period (about 7 weeks) and thereafter the Fe-NTA injection for mice of both sexes was started again and carried on for a specified period. On the day after the last injection, the animals were sacrificed with a narcotic.

Ninety-seven offspring resulting from reciprocal crossing of 26 ICR/JCL mice of both sexes were sex-segregated in separate cages at weaning and 6 weeks after birth were given 126 to 502 intraperitoneal injections of Fe-NTA and on the day after the last injection, all the animals were necropsied.

All the parental mice receiving 30 to 35 injections of Fe-NTA disclosed glycosuria followed by ketonuria and "hemochromatosis" was brought about with more than 120 injections of Fe-NTA, but no amyloidosis occurred in any organs.

Almost all the F₁ mice receiving 30–35 injections of Fe-NTA revealed glycosuria followed by ketonuria to a less or greater degree, although light and electron microscopical examinations of islets of Langerhans of the pancreas showed no discernible changes of the beta cell granules. When given more than 120 injections of Fe-NTA, the offspring developed generalized severe amyloidosis accompanied by slight proteinuria possibly due to massive amyloid deposits in the kidney.

There was widespread erosion or ulcer localized on the skin surrounding both or one side of ears and eyes (Fig. 1).

For the purpose of identifying amyloid substance paraffin sections were stained with the alkaline congo red method (14) and observed in polarized light. Amyloid fibers, 75–100Å in width, were identified by the electron microscopy.

FIG. 1. The gross appearance of the amyloidotic F₁ mouse given 383 injections of Fe-NTA. Note widespread erosion or ulcer (arrows) localized on the skin around its ears and eyes.

Use was made of the Wright *et al.* method (22) which proved very useful in subclassifying amyloid substance deposited on formalin-fixed paraffin sections. After pretreatment with a high concentration (2.5%) solution of permanganate, though it showed a tendency to invade tissue sections to some extent, amyloid-laden sections from various organs such as liver, spleen, heart and kidney were found to lose the positive reaction of both the congo red stain and the same stain viewed in a polarized light for amyloid, this suggesting that these amyloid substances have been derived from protein AA (5).

The population of the 13 mouse families designating “F₁ amyloidosis” were all together in Fig. 2 and in addition, the amyloidotic F₁ mice were shown to have amyloid at an incidence of 69% (67 of 97 mice). Concerning the sex difference in the incidence of amyloidosis occurring in the nonsexual organs of the F₁ mice, as in Fig. 2, females revealed the incidence of approximately 70.2% (40 of 57 mice) showing a 4 percentage increase as compared to the incidence of approximately 67.5% (27 of 40 mice) of amyloidosis in males, this implying that the sex difference examined was not statistically significant ($p < 0.05$). In addition, amyloid deposits were shown to occur with equal severity in both sexes of the F₁ mice. None of the F₁ mice given less than 99 injections of Fe-NTA were found to develop amyloidosis.

The incidence of amyloid deposits in the amyloidotic F₁ mice varied according to the distribution in different organs listed in Table 1. Of the 67 amyloidotic F₁ mice 21 (31%) demonstrated a high degree of amyloidosis of the liver; 29 (43%), spleen and 23 (31%), kidney. Of 67 F₁ mice 28 (42%) developed a moderate

FIG. 2. Diagrams of occurrence of amyloidosis in population of the families shown as a new model for experimental amyloidogenesis.

TABLE 1. *Organ distribution of amyloidosis in 67 amyloidotic F₁ mice*

Organ	‡‡	‡‡	+	The total No. of amyloidotic mice	%
Liver	21	28	18	67	100
Spleen	29	26	12	67	100
Heart	3	18	28	49	73
Kidney	23	19	18	60	90
Pancreas	0	11	16	27	40
Lung	0	2	7	9	13
Adrenal gland	9	11	9	29	43
Digestive tract	10	15	30	55	82
Tongue	0	5	13	18	28

Symbols (++, +, +) represent the degree of amyloid deposits ++=marked; +=mild; +=slight
 Notes: None of 43 mice examined showed amyloid deposits in the brain.

degree of amyloid deposits in the liver; 26 (39%), spleen; 18 (27%), heart and 19 (18%), kidney. The F₁ mice showed varying incidences of amyloid deposits in other various organs. No cerebral involvement of amyloidosis was seen in any of the 43 F₁ mice examined.

Numerous tingible body macrophages were light- and electron microscopically (Fig. 3) at a high incidence found in the splenic germinal center of the amyloidotic F₁ mice.

Electron microscopic examination of the F₁ mice.

Livers. There were massive amyloid deposits filling up the dilated space of Disse and a "stellate formation" (8) of amyloid or "amyloid star" where massive amyloid bundles consisting of well-oriented amyloid fibrils ranging between 80Å and 100Å in width were seen to be arrayed perpendicularly to the Kupffer cell line. Furthermore, bundles of well-oriented amyloid fibrils were observed to be radiating outward from the surface of cytoplasmic invaginations of the Kupffer cells.

Spleen. In the marginal zone one can see macrophages, microvilli of which

FIG. 3. Electron micrograph of the germinal center of the spleen from the amyloidotic F₁ mouse given 211 injections of Fe-NTA. Note a number of tingible bodies (arrows) phagocytized by a tingible body macrophage. N: nucleus of the same macrophage. $\times 6,600$

FIG. 4. Electron micrograph of the amyloid-laden liver of the F₁ mouse receiving 211 injections of Fe-NTA.

Note a Kupffer cell (K₁) attached to a lymphocyte (Ly) with cellular processes (arrow heads) at several spots.

Abundant tufts of amyloid fibrils (AF) are being radiated outward from the intricate, small cytoplasmic invaginations (arrows) of Kupffer cells (K₁ and K₂). H: hepatocytes.
×6,000

are radiating outward abundant bundles of non-branching, well-oriented amyloid fibrils and reticuloendothelial (RE) cells or dendriform reticular cells which are radiating outward massive bundles of amyloid fibrils from their cytoplasmic invaginations. It is noticed that a "marginal zone cell" attached to adjacent lymphocytes with two cellular junctions is radiating outward tufts of amyloid fibrils from the surface of its cytoplasmic invaginations.

Moreover, close contact of "amyloid-forming cells" such as the Kupffer cell (Fig. 4), RE cells, the dendriform reticular cells (Figs. 5, 6) and the "marginal zone cell" (1, 11, 17) to adjacent lymphocytes was frequently found in the liver and spleen of the amyloidotic F₁ mice, but not in those of their parents.

For the purpose of examining whether or not ICR/JCL mice are sensitive to amyloid induction, 14 mice of the same strain, weighing 19–21 g, were subcutaneously injected with 0.5 ml of 5% azocasein in 0.25% NaOH, 6 times per week for 2 months.

Of 6 males, 5 (about 83%) and of 8 females, 7 (about 87%) revealed severe splenic amyloidosis, while the animals given less than 27 injections of azocasein scarcely did. These data suggest that this strain is relatively resistant to amyloid induction, in comparison with the results of Janigan *et al.* (10) that inbred mice

FIG. 5. Electron micrograph of a portion of a dendriform reticular cell (DR) in the marginal zone of the spleen from the F₁ mouse receiving 167 injections of Fe-NTA.

Note thick tufts of interwinding, non-branching amyloid fibrils (AF) radiating outward from the surface of numerous cytoplasmic invaginations. The close proximity (arrowheads) of the DR cell to a lymphocyte (Ly) is noted. Abundant free polyribosomes are shown by thin arrows. N: nucleus of the DR cell, p: prominently twisted cytoplasmic projections, rER: dilated rough endoplasmic reticulum. $\times 10,380$

which are susceptible to amyloid induction can develop generalized amyloidosis with only 9 daily injections of casein.

According to Roitt (15), an immunologically "virgin" rat, i.e., one which has had no previous contact with a specific antigen, may be inoculated with small lymphocytes from a rat which has already given a primary response to that antigen. Challenge of the recipient rat with antigen leads to a secondary type response with the rapid production of high-titre antibodies. If the recipient had not been injected with small lymphocytes from the "primed" donor, a primary response with the relatively slow development of lower titre antibodies would have been seen. Thus the small lymphocytes carry the memory of the first contact with antigen. The following amyloid transfer experiments have been carried on in order to explore whether the above viewpoint of Roitt (15) is applicable to the mechanisms of development of "F₁ amyloidosis".

Amyloid transfer experiments. Group A. Donor treatment. The offspring concerned were obtained by reciprocal crossing of a male given 304 intraperitoneal injections of Fe-NTA and a female given 302 intraperitoneal injections of Fe-NTA.

One of the offspring receiving 124 injections of Fe-NTA through the tail vein,

FIG. 6. Electron micrograph of another dendriform reticular cell (DR) in the marginal zone of the amyloid-laden spleen from the F_1 mouse after 126 injections of Fe-NTA. Note abundant bundles of non-branching, well-oriented amyloid fibrils (AF) radiating outward from the surface of numerous cytoplasmic invaginations (long arrows). The same cell contains a large distorted-shaped nucleus (N) with chromatin situated peripherally, prominent vesicular Golgi zone (G), dilated rough endoplasmic reticulum (rER) and a prominent lysosome (lys) in the cytoplasmic matrix. Notice close contact (short arrows) of well-developed cytoplasmic projections (P) of the dendriform reticular cell (DR) to two lymphocytes (Ly_1 and Ly_2). $\times 11,500$

6 weeks after birth, developed " F_1 amyloidosis". Donor spleens were removed and homogenized. Recipient treatment. 0.5 ml of the spleen suspension containing 10^8 nucleated cells was injected into recipients through the tail vein. All recipients were then given with 12 subcutaneous injections of nitrogen mustard-N-oxide hydrochloride (Yoshitomi Seiyaku Co.-made Nitromin) every second day and the day after the last injection the animals were sacrificed. Results. Of 9 recipients 3 (about 33%) revealed generalized severe amyloidosis and the other 6 showed no amyloidosis. Group B. Donor treatment. Each of 9 mice was given 90 intraperitoneal injections of Fe-NTA 6 times a week and the day after the last injection all were sacrificed. Donor spleens all were removed and homogenized. Recipient treatment. 0.5 ml of the spleen suspension containing 10^8 nucleated cells was injected into each of the recipients through the tail vein. All recipients were then injected with 120 injections of Fe-NTA 6 times a week and the day after the last injection all the animals were sacrificed. Results. Of 9 recipients 4 (about 44%) revealed generalized moderate amyloidosis and the other 5 showed no amyloidosis.

These results of groups A and B suggest that Fe-NTA-carrier conjugate has an immunogenicity.

Fe-NTA (Mol. wt. approximately 244) itself can not be an effective immunogen which usually needs to be more than 5,000 in molecular weight, but it was demonstrated that iron chelated by nitrilotriacetic acid (NTA) combines much more rapidly with transferrin (Mol. wt. approximately 7,000) than with ferric citrate (4). It also is noted that Fe-NTA injected has specific biological effects characterized mainly by extraordinarily enhanced uptake by Chang cells of ferric iron from Fe-NTA (up to 30 times than from transferrin) when measured with ⁵⁹Fe (21).

On the other hand, it was strongly suggested that iron in the blood of pregnant animals would reach the fetus (or newborn) via the placenta (or milk) (1, 6, 9, 12, 20). But considering that the Fe-NTA injection was stopped for about 7 weeks from crossing to weaning, a relative minimum of iron derived from Fe-NTA injected is supposed to remain in pregnant mouse blood. According to Nossal (13), immunological memory phenomenon is best shown with antigens which cause practically no detectable primary immune response because of very low inherent immunogenicity or of a very small dose of the antigens administered.

Tingible body macrophages observed frequently in the germinal center of the amyloidotic spleen of the F₁ mice may imply, as reported by Schwarzendruber *et al.* (18), an antibody-stimulated status.

There are numerous reports manifesting the interaction between macrophages or dendritic cells (19) and adjacent lymphocytes under amyloid-free conditions. Most of the above reports strongly suggest that such cell clusters may be required for antibody production. There are, however, scarcely any reports showing that "antigen-representing cells" such as Kupffer cells (Fig. 4,) dendriform reticular cells (Fig. 5) and "marginal zone cells" are radiating outward amyloid fibrils from the surface of cellular invaginations, this signifying the appearance of an immune response which might be involved in the pathogenesis of "F₁ amyloidosis."

Alternatively, Scheinberg *et al.* (16) evidenced that specific materials such as casein used to induce amyloidosis are potent macrophage activators invoking the initial step in the development of amyloidosis. This suggests that Fe-NTA also might be one of macrophage activators in the F₁ mice. The above analysis allow us a brief speculation that since multiple injections of Fe-NTA stimulate chronically, and proliferate selectively F₁ mouse clones to respond better to Fe-NTA than in the case of their parents, supposed immunological effects transmitted from mother given the Fe-NTA injection, together with macrophage dysfunction, might have been, involved in amyloidosis in the F₁ mice receiving the Fe-NTA injection.

Moreover, since a memory state may last for a long period after the antibody from a primary immune response elicited has declined within a relatively short period, the individual which is "primed" can give a more rapid secondary immune response to a subsequent exposure to the same antigen (7). Thus, it is possible to assume that Fe-NTA administered would have caused a secondary immune response as a second "shot" in the offspring which have acquired a state of memory for Fe-NTA-carrier conjugate as a result of receiving iron conjugate in their perinatal period from a mother treated with the same agent.

In order to obtain further evidence for the above assumption the following several "fosterage experiments" have been planned and initiated in order. Preliminary reports.

Group 1. The offspring from the parental mice both of which received 96 injections

of Fe-NTA before crossing were intraperitoneally injected with Fe-NTA, 150 to 163 times (6 times per week), since lactation by an untreated puerperal wet nurse. Result. Of 9 offspring 4 developed amyloidosis.

Group 2. The offspring from untreated parental mice were administered 189 to 195 injections of Fe-NTA since lactation by a puerperal wet nurse receiving previous 96 injections of Fe-NTA. Result. None of 9 offspring developed amyloidosis.

Group 3. The offspring from the parental mice both of which received 96 injections of Fe-NTA before crossing were administered 174 or 195 injections of Fe-NTA since lactation by an untreated puerperal nurse. Result. Of 9 offspring 3 developed amyloidosis.

Group 4. The offspring from untreated parental mice were administered 163 or 169 injections of Fe-NTA since lactation by a puerperal wet nurse receiving 99 injections of Fe-NTA. Result. Of 9 offspring only one developed amyloidosis.

The results of groups 1, 2, 3, and 4 are favorable for the supposition that the antigen would reach the fetus mainly via the placenta rather than via milk.

Group 5. The offspring from reciprocal crossing between a female receiving 99 injections of Fe-NTA before crossing and an untreated male were administered with 150 or 158 injections of Fe-NTA. Result. Of 10 offspring 3 developed amyloidosis.

Group 6. The offspring from reciprocal crossing between an untreated female and a male receiving 99 injections of Fe-NTA before crossing was administered 175 injections of Fe-NTA. Result. None of 7 offspring developed amyloidosis.

The results of groups 5 and 6 are favorable for the supposition that the antigen would be transferred to the offspring via mother.

Group 7. None of the offspring from the parental mice both of which received 100 injections of Fe-NTA were injected with Fe-NTA. Result. None of 8 offspring developed amyloidosis, this suggesting that Fe-NTA injection to the offspring is a crucial step in the development of "F₁ amyloidosis".

Moreover, immunological memory persists for several months in the mouse after the first response to the antigen, even when serum antibody concentration has become very low or even nil, meaning below the detection threshold (Bach) (2). It also is possible to assume that a very small amount of the antibody remaining in the F₁ mouse blood as a result of transmission into the fetus from the mother given Fe-NTA chronically might have responded well with the same antigen as in their maternal mice, inducing a secondary immune response considered to play a role in the pathogenesis of "F₁ amyloidosis."

In summary, Fe-NTA-induced "F₁ amyloidosis" has been first reported in this paper and its pathogenesis is discussed, much yet remaining to be investigated.

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