Dialysis Associated Auto-Antibodies

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Summary

The incidence of anti-nuclear antibodies (ANA), non-specific cold (NS) and anti-N-like cold haemagglutinins (CN) were studied in 288 dialysis patients divided into four groups. Thirty patients on long term peritoneal dialysis had a 10% incidence of ANA, 27% NS and no CN. Sixty-seven patients on 3x week haemodialysis with single use non-formalin sterilised dialysers had a 11% incidence of ANA and 30% NS and no CN. Forty patients on 3x week single use formalin sterilised dialysers had a 9% incidence of ANA and 12% NS and 20% CN. One hundred and fifty-one patients on 3x week re-used formalin sterilised dialysis had a 10% incidence of ANA, 14% NS and 19% CN. Formalin sterilisation was associated with the presence of an anti-N-like phenomenon. In vitro it was suggested that this was an artefact occurring in patients who had preformed non-specific cold agglutinins, which the presence of free formalin in the plasma rendered more specific for red cells bearing the N antigen. The mechanism of the anti-nuclear antibody formation is unknown; its presence in peritoneal dialysis patients is not explained by reinjection of white cell nuclei from the dialysis membrane. It was not particularly associated with re-use. It was concluded that re-use is medically sound and in the absence of chronic toxicity studies formalin should continue to be used as a chemical sterilant.

In 1972, Howell and Perkins reported the presence of a quasi specific red cell cold agglutinin, which although exhibiting non-specific qualities, showed a preference for agglutinating with red cells of the NN blood group. They called this antibody, anti-N-like to distinguish it from the extremely rare true anti-N antibody. Anti-N-like antibodies were found in the sera of 12 out of 416 long term haemodialysis patients. They postulated that formalin might be associated
with the production of this antibody, especially in three patients who had an
NN homozygous blood group, and suggested that formalin might alter the N
antigen rendering it immunogenic. Since then several reports appear to have
confirmed that dialysis patients dialysed either with re-used or once used formalin
sterilised dialysers have a 30–50% incidence of anti-N-like antibodies. Its
occurrence has never been reported in patients dialysed with non formalin
However, Harrison et al (1975) also reported a 32% incidence of non-specific
cold agglutinins in their non-formalin dialysed patients, and Payne et al, 1973,
found a 48% incidence of non-specific cold agglutinins in haemodialysis patients
in the Los Angeles area.

More recently, Nolph et al (1976) has described an increased incidence of
anti-nuclear antibodies in haemodialysis patients, attributed to the reinjection of
white cell nuclei during haemodialysis.

The possibility that both these phenomena might be increased in a dialysis
population who re-used formalin sterilised dialysers, prompted us to determine
the incidence of these phenomena in the dialysis population of the Languedoc-
Roussillon region. In addition, we looked for the presence of anti-DNA anti-
bodies, and performed several experiments in vitro to try and elucidate the
nature of the formalin action on haemagglutination.

MATERIAL

Two hundred and eighty-eight long term dialysis patients were studied, they
were divided into four groups:

1. Peritoneal dialysis 30 patients
2. Haemodialysis without formalin sterilised dialysers 67 patients
3. Haemodialysis with once used formalin sterilised
dialysers 40 patients
4. Haemodialysis with re-used formalin sterilised
dialysers 151 patients

In addition fresh drawn sera were studied from 50 healthy volunteer blood
donors. The time of the study was the winter and spring of 1975/1976.

All patients had been in each group for at least six months at the time of
the study.

METHODS

Ten ml of whole blood was drawn from each patient and allowed to clot in a
glass tube. The serum was removed and was either examined immediately or
stored in a deep freeze. The relationship of the last dialysis to the time of the
sample was not noted, but no immediate post-dialysis samples were taken to
avoid heparin reducing the serum yield.

Red cell cold agglutinins were determined using the tube technique without centrifugation. Sera were screened at 4°C, 20°C and 37°C after incubation for 1 h at each temperature. The results were graded from 0 to ++++. If the cold reaction was of equal intensity for MM, MN and NN cells it was considered non-specific. On warming, if the preference for the N antigen increased it was considered an anti-N-like phenomenon. In the latter case, the patients own cells were also used after the determination of the MN blood group.

Anti-nuclear and anti-DNA antibodies were studied by an indirect immunofluorescent technique (Seignalet, 1971). The incidence of positive reaction for anti-nuclear antibodies is 0.3% in a healthy population with this method.

Haemagglutination Inhibition in vitro studies

1. Ten ml 4% formalin diluted in saline or distilled water was added to 0.5 ml 3% washed MM red cell suspension. The mixture was centrifuged at 2000 rpm for 10 minutes. Control suspensions in saline or distilled water were also run. The sediment was examined microscopically. The formalin-treated red cells, either stroma (formalin/water), or intact red cells (formalin/saline) were washed six times and then incubated at 4°C for 12 hours with a serum known to contain a high titre anti-N-like antibody, which had been previously unreactive to the MM cells used. The supernatant was removed and the anti-N-like antibody titre was remeasured.

   It was noticed that formalin had an ability to agglutinate red cells independently of antibody, and to enhance weak agglutination reactions. It was therefore considered that all in vitro tests between red cells and sera containing cold agglutinins depending on a direct haemagglutination where formalin had been added were invalid because of this artefact.

2. Studies of the effect of dialysis on sera containing ++++ anti-N-like antibodies. A ++++ anti-N-like serum was dialysed using cellophane against an iso-osmotic solution at 4°C for 4 h. Negative serum was used as a control. The sera were then restudied for their cold agglutinating properties.

RESULTS

The results are summarised in Tables I and II.

Red cell cold agglutinins

In vivo and anti-nuclear and anti-DNA antibodies.

Group 1 Peritoneal dialysis. Eight out of 30 patients (27%) had non-specific cold agglutinins present. In two out of 20 patients (10%) anti-nuclear antibodies were found at a titre of 1/4. No anti-N-like antibodies were found, nor anti-DNA. The
TABLE 1 Incidence of red cell cold agglutinins in populations

<table>
<thead>
<tr>
<th>Group</th>
<th>Nos. of patients</th>
<th>R.B.C. cold agglutinins</th>
<th>Anti-N-like</th>
<th>Non-specific</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nos.</td>
<td>%</td>
<td>Nos.</td>
<td>%</td>
</tr>
<tr>
<td>1 (PD)</td>
<td>30 (26.9±3.4)</td>
<td>8 (25.4±4.1)</td>
<td>27</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2 (HD)</td>
<td>67 (26.9±4.5)</td>
<td>20 (27.4±4.8)</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3 (HDF)</td>
<td>40 (26.7±5.4)</td>
<td>13 (26.0±3.9)</td>
<td>32</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>4 (HDFR)</td>
<td>151 (27.0±5.1)</td>
<td>51 (27.0±6.0)</td>
<td>33</td>
<td>29</td>
<td>19</td>
</tr>
<tr>
<td>Total</td>
<td>288</td>
<td>92</td>
<td>32</td>
<td>37</td>
<td>13</td>
</tr>
<tr>
<td>Controls</td>
<td>50</td>
<td>11</td>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1 p < 0.05 between incidence of cold agglutinins in dialysis population as a whole and controls.
2 There was no significant difference between the incidence of cold agglutinins in the four dialysis groups.
3 Anti-N-like cold agglutinins were only found in patients dialysed with formalin sterilised dialysers. There was no significant difference in incidence between once-used and re-used populations.
4 Haematocrit in brackets = mean ± S.D. for each group.

Mean haematocrit for this group was 26.9% ± 3.4. There was no significant difference between the haematocrit of the positive non-specific cold agglutinin patients and the rest of the group.

**Group 2** Haemodialysis with once-used non-formalin sterilised dialysers. Non-specific cold agglutinins were found in 20/67 patients (30%). No anti-N-like antibodies were detected. 5/45 patients (11%) had a 1/4 positive anti-nuclear antibody. No patient had a positive anti-DNA. The mean haematocrit was 26.9% ± 4.5. There was no significant difference between the mean haematocrit of the positive cold agglutinin patients and the negatives.

**Group 3** Haemodialysis with once-used formalin sterilised dialysers. Red cell cold agglutinins were detected in 13/40 patients (32%). Eight patients (20%) had the anti-N-like phenomenon and five (12%) had non-specific antibodies. 3/33 patients (9%) had a 1/4 anti-nuclear antibody. No patient had a positive anti-DNA. The mean haematocrit was 26.7% ± 5.4 and did not differ significantly between the group as a whole, the anti-N-like patients, the non-specific patients or those who were negative.
TABLE II Incidence of Anti-Nuclear and anti-DNA antibodies

<table>
<thead>
<tr>
<th>Group</th>
<th>Nos. of patients</th>
<th>Anti-Nuclear</th>
<th></th>
<th></th>
<th>Anti-DNA</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1/4</td>
<td>1/16</td>
<td>%</td>
<td>1/4</td>
<td>1/16</td>
<td>1/256</td>
</tr>
<tr>
<td>1 (PD)</td>
<td>20</td>
<td>2</td>
<td>-</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 (HD)</td>
<td>45</td>
<td>5</td>
<td>-</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 (HDF)</td>
<td>33</td>
<td>3</td>
<td>-</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 (HDFR)</td>
<td>109</td>
<td>10</td>
<td>1x</td>
<td>10</td>
<td>2</td>
<td>1x</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>207</td>
<td>20</td>
<td>10</td>
<td></td>
<td>2</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

The incidence of Anti-nuclear antibodies was not significantly different between any group.

x This patient had S.L.E.

Group 4 Haemodialysis with re-used formalin sterilised dialysers. Red cell cold agglutinins were detected in 51/151 patients (33%). Twenty-nine (19%) patients had an anti-N-like cold agglutinin and 22 (14%) had non-specific antibodies. 11/109 (10%) patients had a positive anti-nuclear antibody (10 at a titre of 1/4 and 1 at a titre of 1/16). Three of these positive patients had a positive anti-DNA (two at a titre of 1/4 and one at a titre of 1/256). This latter patient had a primary renal diagnosis, confirmed by biopsy, of systemic lupus erythematosus. The mean haematocrit was 27.0% ± 5.1. There was no significant difference between the haematocrit of the patients with anti-N-like antibodies, with non-specific antibodies and the antibody-negative patients.

There was no significant difference between the mean haematocrit of any group studied. The incidence of red cell cold agglutinins is probably identical in each group. However, only the formalin once-used and re-used groups studies showed the anti-N like phenomenon.

In vitro

Formalin 4% diluted in saline did not haemolyse red cells, but caused these red cells to agglutinate spontaneously. The haemagglutination-inhibition test using MM cells incubated with formalin and a high titre anti-N-like serum was negative. Saturation of NN cells with commercially available specific anti-N antibody did not prevent these cells, after washing, from agglutinating with anti-N-like serum. This observation casts doubts on the specificity of the anti-N-like antigen site, as clearly, when the N site was blocked the anti-N-like antibody was still absorbed by the red cell envelope.
In vitro antibody studies

The effect of dialysis on a +++ MN ++++ NN — MM reaction was to reduce the reaction to ± MN ± NN — MM.

It was also noted that several anti-N-like patients have become negative in the summer as have several non-specific cold agglutinin reactions.

DISCUSSION

Antinuclear antibodies

The increased incidence of anti-nuclear antibodies in our dialysis population confirms a previous report (Nolph et al, 1976).

However, the fact that there was no difference in incidence between the re-use group and the other groups suggests that re-use is not responsible for this phenomenon. Nolph et al (1976) suggested that leucocyte nuclear debris which he has shown to be attached to the dialysis membrane during haemodialysis might become detached and re-injected into the patient, causing an auto-immune reaction. The failure to find anti-DNA antibodies in the majority of the antinuclear positive patients casts some doubt on this interpretation. Further, the presence of this weak positive in peritoneal dialysis patients demands an alternative explanation. In the absence of longitudinal studies and in the relatively non-specific nature of the antinuclear reaction, one must reserve judgement on the mechanism. Nolph used an haemagglutination technique which is more sensitive but less specific than immunofluorescence. This might explain the 30% of anti-nuclear antibodies in his dialysis population. He found no difference between non re-users and re-users but did not study patients longitudinally. In addition, the underlying renal disease noted in his series, and in ours, lacks histological confirmation in the majority of cases.

Anti-N-like phenomenon

The increased incidence of non-specific cold agglutinins in a dialysis population has been attributed to the higher incidence of chronic viral infections (Payne et al, 1973). The striking similarity in incidence of cold agglutinins (non-specific or anti-N-like) of 30% in all our groups suggested, perhaps, that these patients had preformed cold agglutinins and that in some way formalin was changing the specificity of the in vitro reaction. Ogden et al (1973) has shown, using a sensitive colorimetric test for formalin (Hantzsch reaction), that patients connected to formalin sterilised dialysers, where the venous effluent was negative to a clinistest tablet after saline rinsing, all received trace quantities of formalin during dialysis. More recently, (Odgen, 1976, personal communication) using 14C formalin, has shown an incomplete recovery of 14C from a dialyser sterilised with radioactive formalin. This was in spite of washing both compartments with
literally hundreds of litres of saline. In addition an aliquot of each rinse contained radioactivity. He concludes that the patient will receive trace quantities of formalin with each dialysis if a formalin sterilised dialyser is used.

Møller et al (1976) using gas chromatography, could detect formalin in trace quantities in the third washing of a REDY SYSTEM sterilised in 1% formalin, and again doubts if all formalin can be removed from an apparatus sterilised in formalin.

If these observations are confirmed, it is reasonable to assume that many dialysis patients have been receiving trace quantities of formalin with every dialysis. However, there are no published chronic toxicity studies on formalin in man or animals.

It is known that formalin is oxidised in the dog to methyl alcohol and formic acid and the latter is then metabolised to CO fragments, also that the plasma half-life of formalin is 90 minutes, but that it is detectable in red cells up to six hours in the dog.

Our chance observation that formalin agglutinates red cells spontaneously and the observation by Fassbinder et al (1976) that it enhanced in vitro cold agglutinations of the anti-N-like type suggested to us that the anti-N-like phenomenon was an artefact due to the presence of free formalin in the plasma of patients dialysed against a formalin sterilised dialyser who had previously preformed non-specific antibodies. In addition, the disappearance of the anti-N-like antibody in some patients in summer, whilst dialysis techniques have remained constant, indicates the necessity of a longitudinal study to clarify this problem.

Our observation on the weakening of the anti-N-like phenomenon by dialysis of positive serum supports the artefact nature of the phenomenon, if we assume dialysis removed free formalin.

The clinical importance of cold agglutinins is dubious, apart from the risk of massive agglutination in a cold transplanted kidney (Belzer et al, 1971). It was suggested that these antibodies should be looked for immediately before transplantation, and the donor kidney rewarmed. The absence of any difference in haematocrit between our four groups suggests that the anti-N-like phenomenon does not play an important haemolytic role and contribute to the anaemia of the dialysis population no more than would the random occurrence of non-specific cold agglutinins. Yet the latter, known to exist for years, has never been incriminated as a cause of haemolytic anaemia in the dialysis population. Perhaps the fact that the anti-N-like phenomenon is associated with re-use has prompted some authors (Kaehny et al, 1975, Harrison et al, 1975) to use this as a warning against re-use. We refute this argument and insist that until chronic toxicity studies have demonstrated a toxic effect from trace quantities of parenteral formalin, it should continue to be used as a sterilant. One could draw an analogy with the toxic effects of phthalate plasticisers which leach out of PVC blood tubing sets during dialysis (Neergard et al, 1971, Geertz et al, 1974) but which
“faute de mieux” are still used for all extracorporeal circulations today. The search for a more efficient non-toxic chemical sterilant is justified on social grounds as formalin is a dangerous toxic substance in high concentration, but its trace presence in the human body has not yet been shown to be toxic.

We conclude that in the light of present knowledge neither the anti-nuclear nor the anti-N-like phenomena of dialysis justify the name auto-antibodies and suggest further longitudinal studies in man, coupled with $^{14}$C formalin studies in animals, are necessary to elucidate these findings.

References

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Open Discussion

M ROBERTS (Los Angeles) Formaldehyde has been given intravenously to humans as a 0.4% solution in 500 ml quantities over a 2-hour period without any acute toxicity. This unpublished work was done in the 1950's. The formaldehyde solution was given to patients in severe hepatic coma in an attempt to reverse the coma by forming a non-toxic addition compound with ammonia. No beneficial results were noted.

ROBINSON (Birmingham) I would just like to direct one question to Dr Shaldon. I do not think it affects in any way the validity of conclusions about anti-N-like antibodies, but turning to some of the other non-specific antibodies, I wonder whether a better control group would not be healthy blood donors, but patients with renal disease who are not on dialysis, because we do find quite a high incidence of, for example, antinuclear antibodies in patients with glomerulonephritis.
SHALDON I take your point. However, among the population available to us to study we did not have a sufficiently large number of renal failure patients who were not on dialysis.

BRUNNER (Basel) It seems that certain patients develop anaphylactoid reactions due to formalin, which they get even after good rinsing of dialysers. Have you observed such patients and what could you do about it?

SHALDON I think the question of whether a patient is sensitive to anything in a dialysis system, in the extracorporeal circulation, can only be proved by elimination. I imagine that you have patients as described, you take the formalin out of the system, and then you no longer have the reaction. I think that this is a problem common to many dialysis materials. We have patients who are sensitive to the polyacrylonitrile membrane. There are patients who are sensitive, apparently, to the cuprophane membrane. There are undoubtedly plasticisers leaching out of the PVC continuously, and probably giving trace intoxications; so that I think all these things need to be looked at. But I don't think the objective of our study was to stop dialysis whilst we eliminated all trace contaminants.

CATTELL (London) Could we possibly, Mr Chairman, ask Dr Fassbinder whether he has any observations to make on the different findings by the two groups? Dr Fassbinder on the one hand finds this correlation with formaldehyde sterilisation, which Dr Shaldon has not observed.

SHALDON On the contrary, I am sorry, I must have expressed myself extremely badly. That's the one thing we do agree on. We find the anti-N-like phenomenon only in patients who have been dialysed against formalin sterilised dialysers.

CATTELL I thought also in your peritoneal dialysis group?

SHALDON No, we found, there, non-specific cold agglutinins, not the anti-N-like phenomenon.

CATTELL I beg your pardon.

CHAIRMAN Dr Fassbinder, do you have a comment?

FASSBINDER No, concerning this point we have the same results in Montpellier and Frankfurt.