

### CIRCULATING ANTIBODIES TO OVINE AND BOVINE IMMUNOGLOBULIN IN HEALTHY SUBJECTS: A HAZARD FOR IMMUNOASSAYS

SIR,—During the development of a sandwich immunoradiometric assay (IRMA) for measuring  $\alpha$ -fetoprotein (AFP) in sera from both pregnant and non-pregnant subjects, some samples from blood donors of either sex exhibited marked positive bias when compared with the levels obtained in a conventional inhibition-type labelled antigen radioimmunoassay (RIA). The IRMA system involved incubation of 100  $\mu$ l serum containing the antigen (Ag) with  $^{125}$ I-antibody (\*Ab) for 16 h and separation of the \*Ab-Ag complex by reaction for 3 h with excess of an Ab/'Sepharose 4B' coupled reagent. The antibodies for both sides of the sandwich were from the same sheep antiserum to AFP. The RIA used the same primary antiserum and a donkey anti-sheep IgG serum was used for the second antibody precipitation separation system. The working range of the IRMA (2.5–80% bound where 100% is the computer determined upper asymptote, generally equal to 60–80% of the added \*Ab) was 200–250 U/l: that of the RIA (85–15% of the binding of tracer in the absence of unlabelled AFP) was 600–1200 U/l for 100  $\mu$ l serum sample in each case.

The anomalous sera gave response curves in the IRMA which were steeper than the AFP standard curves and some such samples gave higher responses than could be achieved with excess of AFP. The following evidence supports the hypothesis that the anomalous responses were due to the presence of circulating antibodies to sheep immunoglobulins, which, if present, would, as bifunctional reagents, be well able to link \*Ab with solid-phase linked Ab and so "read" as antigen. The anomalous activity:

- (1) co-eluted with IgG (3 sera tested) from 'Sephadex G150', whereas AFP is eluted later with albumin (cord serum and two pregnancy serum pools tested);
- (2) migrated 5 mm towards the anode whereas AFP migrated 15 mm on cellulose acetate electrophoresis in pH 8.6 barbitone;
- (3) was absorbed at neutral pH onto protein A from *Staphylococcus aureus* which had been linked to 'Sepharose' (Pharmacia) and desorbed by glycine buffer at pH 3.0 whereas AFP was unabsorbed;
- (4) was quantitatively inhibited by the presence of non-immune sheep serum at incubation dilutions of 1 in 1000 (11 anomalous + 47 control sera tested), of sheep IgG at 20 mg/l, of non-immune bovine serum at 1 in 5000, bovine IgG 20 mg/l or cow's milk at 1 in 80, but not by non-immune rabbit or pig serum at 1 in 20, or horse serum at 1 in 100;
- (5) gave markedly false high values in a sandwich assay for human thyroid stimulating hormone (h-TSH) when the \*Ab was prepared from a sheep antiserum (to h-TSH) but *not* when it was derived from a rabbit antiserum to h-TSH. The former reaction was quantitatively inhibited in the presence of non-immune sheep serum.

Confirmation was obtained when  $^{125}$ I-AFP was preincubated with sheep anti-AFP and then with anomalous and non-anomalous sera (as controls) and both were then treated with excess rabbit antiserum to human IgG. 66% of the tracer was precipitated in the former and 5% in the latter case. There was no such precipitation of tracer when non-immune sheep serum replaced the sheep anti-AFP or when sheep serum was omitted.

There is a marked variation in the "titre" of such circulating antibodies. The prevalence seems to be about 7% (11 of 148 sera tested) in the Edinburgh population of blood donors; 9/112 were from men, 2/36 from women. This rate exceeds that which would be expected if the immunisation was related to occupation—i.e., shepherds, slaughtermen, and butchers and, being apparently equal between the sexes, is unlikely to be related to handling of raw meat (a predominantly female activity outside the above occupations). These considerations favour the suggestion that immunisation may be via the gut: the favourable differential inhibition by bovine serum suggests bovine immunoglobulin as the principal immunogen, though clearly there is marked cross-reactivity with ovine immunoglobulin. Since cow's milk is widely ingested in uncooked form, it may well be the most common means of immunisation. Preliminary evidence suggests that there are also circulating antibodies to ovine serum albumin and perhaps to other serum proteins.

A sensitive two-site sandwich assay is exquisitely sensitive to the presence of such antibodies. A less sensitive 3 h sandwich assay (\*Ab, Ag present in only 25  $\mu$ l of serum, and solid-phase antibody all

added together), was affected by fewer samples; in other words the ratio of patient serum (and hence the amount of antibody to sheep immunoglobulin) to sheep immunoglobulin is important. Most inhibition-type radioimmunoassays were little affected, particularly when the above ratio was low and the incubation short, as in assays using sheep antisera to thyroxine, triiodothyroxine, oestriol, and human placental lactogen which are used for routine clinical purposes in Edinburgh. However, a sensitive RIA for AFP which used a 16 h primary incubation in 50% patient serum did give positive bias with the highest titres of anomalous sera.

In a recovery experiment AFP standard was added, 90 000 U/l, to each of twenty individual non-pregnant female donor sera, and samples sent to ten different laboratories involved in routine screening measurements of maternal serum AFP who used a variety of different procedures. One of the sera was anomalous by the above criteria—i.e., it contained a high titre of antibodies to sheep immunoglobulin. Recoveries of AFP from this serum were consistently low (mean  $86.0 \pm 4.5\%$  SD) in inhibition assays employing polyethyleneglycol (PEG), double antibody and PEG enhanced double antibody separation; recovery was  $97.8 \pm 5.0\%$  for the other 19 sera. The mechanism responsible for this negative bias is unclear. Presumably the existence of an antibody complex before addition of the separation reagents can enhance precipitation of the bound fraction. This level of interference would go unnoticed, but it could result in a patient carrying a fetus with a neural tube defect being missed in a screening programme. However, the two laboratories who used a solid-phase double antibody separation system reported recoveries in excess of 200% for the anomalous serum (105.9% for the other 19 samples). Occupation of antigenic determinants on the primary antibody presumably renders them unavailable to the solid-phase reagent. Such distortion would, if unrecognised, result in a patient carrying a normal fetus going unnecessarily to amniocentesis. Recovery was accurate when non-immune sheep serum was included in the incubates.

Most sensitive RIA and IRMA have hitherto used antisera raised in rabbits and guinea pigs, and humans are very unlikely to carry circulating antibodies to these animals' immunoglobulins. As such assay procedures go into widespread routine use antisera are, for good economic reasons, increasingly being raised in sheep, and it is important that the possibility of distortion due to this cause be recognised. The remedy is simple and completely effective: if the assay incubates from the outset contain non-immune serum of the species used to raise the anti-serum (whether or not this is labelled), then the distortion is entirely prevented.

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### TOXIC PYRROLIZIDINE ALKALOIDS IN COMFREY

SIR,—Concern has been expressed about possible health risks to people who use the herb comfrey (*Symphytum* spp) as a green vegetable, beverage, or remedy.<sup>1</sup> The leaves and roots of a Japanese sample of *Symphytum officinale* were hepatocarcinogenic in the rat.<sup>2</sup> The herb contains up to eight toxic pyrrolizidine alkaloids,<sup>3</sup> and at least one of these, symphytine, is carcinogenic to rats.<sup>4</sup>

1. Anon. Question and reply. *Br Med J* 1979; **1**: 598.
2. Hirono I, Mori H, Haga M. Carcinogenic activity of *Symphytum officinale*. *J Nat Cancer Inst* 1978; **61**: 865–69.
3. Culvenor CCJ, Clarke M, Edgar JA, Frahn JL, Jago MV, Peterson JE, Smith LW. Structure and toxicity of the alkaloids of Russian comfrey (*Symphytum X uplandicum* Nyman), a medicinal herb and item of human diet. *Experientia* 1980; **36**: 377–79.
4. Hirono I, Haga M, Fujii M, Matsuura S, Matsubara N, Nakayama M, Furuya T, Hikichi M, Takanashi H, Uchida E, Hosaka S, Ueno I. Induction of hepatic tumors in rats by senkirkine and symphytine. *J Nat Cancer Inst* 1979; **63**: 469–71.

We have measured pyrrolizidine alkaloids in the leaves of the hybrid Russian comfrey *S. X uplandicum* (variety Bocking no. 14, grown at Bocking, Essex, and kindly supplied by Mr L. D. Hills of the Henry Doubleday Research Association). Twelve long-established plants were cut back to ground level in early August, 1979. The largest (over 18 cm long) and shortest (under 15 cm) were separately pooled, air-dried in the dark at 15–25°C, milled, and extracted exhaustively with methanol; the intermediate sized leaves were discarded. The alkaloids were isolated and analysed by spectrophotometry.<sup>5,6</sup>

Total alkaloid levels were 0.003% in the largest leaves and 0.049% in the smallest: a 16-fold variation. Subsequently the total new leaf growth from pairs of the same plants were harvested and analysed. Leaves picked after the first 2 weeks contained 0.115% of alkaloids (dry weight) while by mid-September the level had fallen to 0.019% and remained between 0.019 and 0.022% until mid-October (when growth ceased).

For a further analysis, plants were cut back in early April, 1980, then all the leaves from pairs of plants were harvested every 2 weeks, counted, and pooled. The amount of alkaloids in the form of bases and of N-oxides was measured. In one case the total alkaloids were also estimated by a different method,<sup>7</sup> with comparable results. The total amount of alkaloids per leaf remains fairly constant (see table);

## ANALYSIS OF PYRROLIZIDINE ALKALOIDS IN DRIED COMFREY LEAVES

Date (1980)	No. of leaves	Mean leaf weight (g)	% weight alkaloids		Mean total alkaloids per leaf (mg)	N-oxides as % of total alkaloids
			Bases	Bases + N-oxides		
April 13	665	0.17	0.085	0.222	0.37	62
April 14	955	0.23	0.087	0.12*	0.27	28
April 28	315	0.69	0.043	0.048	0.33	10

\*0.13 using alternative method.<sup>7</sup>

as the leaf grows heavier, so the percentage of alkaloids falls and alkaloids in the form of N-oxides are progressively converted to bases. (The base and N-oxide forms of pyrrolizidine alkaloids are likely to have similar toxicity when taken by mouth.<sup>8</sup>) The amounts of alkaloids found are consistent with the range of values previously reported. Thus Culvenor et al.<sup>3</sup> found 0.01–0.15% total alkaloids in different samples of dried comfrey leaves, and Pederson<sup>9</sup> up to 1.97 parts per 1000 (0.2%); however, the age and size of the leaves were not recorded. My measurement show that the highest alkaloid levels are in small, young leaves, especially early in the season.

The possible dangers of ingesting comfrey have been discussed elsewhere.<sup>1,3</sup> People who consider the benefits of comfrey to outweigh the (perhaps slight) risk involved may like to know that large, mature leaves carry the lowest concentrations of toxic alkaloids. Moreover, protein extracted from comfrey should not be harmful: a sample of comfrey protein supplied by Mr Hills proved, as expected, to be completely free of alkaloids. The external use of comfrey preparations should not be hazardous since the alkaloids are converted to toxic metabolites by liver enzymes only after being ingested.<sup>10</sup>

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## ULNAR NERVE LESION AS COMPLICATION OF CIMINO-BRESCIA ARTERIOVENOUS FISTULA

SIR,—Carpal tunnel syndrome is a well-recognised complication of arteriovenous fistulae constructed in the forearm to provide vascular access for chronic intermittent haemodialysis.<sup>1</sup> We wish to report three patients with ulnar nerve lesions in their non-dominant (fistula) arm, a complication of Cimino-Brescia fistulae.

*Case 1.*—A 35-year-old man with chronic renal failure secondary to chronic pyelonephritis had a side-to-side left-radial-artery/cephalic-vein fistula constructed in June, 1979, before starting chronic intermittent haemodialysis. In November, 1979, he received a cadaveric renal transplant which functioned well. 2 months before his admission in June, 1980, with a urinary tract infection he had noticed numbness of the fifth finger and the ulnar border of the fourth finger of his left hand. 2 weeks before admission he had noticed some wasting of the small muscles of his left hand. He had both sensory and motor signs of ulnar nerve palsy. Electromyography confirmed the isolated left ulnar nerve palsy.

*Case 2.*—A 50-year-old man with chronic renal failure secondary to membranous glomerulonephritis began haemodialysis in June, 1979. In July, 1979, a Cimino-Brescia fistula was constructed between his left radial artery and cephalic vein. In June, 1980, he received a cadaveric transplant which functioned well. In July, 1980, wasting of the small muscles of his left hand associated with slight weakness was noted, but there was no sensory loss.

*Case 3.*—A 22-year-old man with chronic renal failure secondary to chronic pyelonephritis had a Cimino-Brescia fistula constructed between his left radial artery and cephalic vein on Nov. 9, 1978, before starting haemodialysis. It thrombosed shortly afterwards, and on Nov. 15 a fistula was constructed between his left brachial artery and antecubital vein. Chronic intermittent haemodialysis was started in January, 1979, and he received a cadaveric renal transplant in February, 1979. One month later left ulnar and medial nerve palsies developed. Ligation of the distal portion of the vein in the left antecubital fossa resulted in some improvement in the power of grip in his left hand. In April, 1979, he required a transplant nephrectomy and he is at present on chronic haemodialysis.

The relation between these three ulnar nerve lesions and the presence of arteriovenous fistulae seems more than fortuitous. Ulnar nerve lesions have been reported in association with metabolic disturbances, but this seems unlikely in these cases since two patients had normal renal function. Mononeuritis may complicate diabetes, amyloid, or polyarteritis nodosa, but there was no evidence of these conditions. Compression of the ulnar nerve at the elbow during anaesthesia has been reported,<sup>2</sup> but the onset of these patients' symptoms was far removed from the time of surgery. Ischaemia of the ulnar nerve has been reported in association with Volkman's ischaemic contracture, but in none of the patients was there any sign of vascular insufficiency in the hand or forearm. The most likely explanation for the ulnar nerve lesions in these patients is the cubital tunnel syndrome. This syndrome, first described by Feindel and Stratford,<sup>3</sup> is secondary to ulnar nerve compression as it passes beneath the fibroaponeurotic arch beneath the humeral and ulnar heads of flexor carpi ulnaris. This opening is especially narrow during elbow flexion.<sup>4</sup> Prolonged immobility of the arm during haemodialysis, especially if the elbow is flexed, may predispose patients on haemodialysis to this condition. More important, however, is the increased volume of the arm as a result of increased flow and the raised venous pressure caused by the fistula. This may cause narrowing of the cubital tunnel. A similar explanation is believed to be the mechanism for carpal tunnel syndrome in haemodialysis patients.<sup>1</sup> The improvement experienced by the third patient following venous ligation would support a diagnosis of cubital tunnel syndrome due to venous hypertension.

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