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Human antibody response to fragments A and B of diphtheria toxin and a synthetic peptide of amino acid residues 141–157 of fragment A

V. Y. PERERA* AND M. J. CORBEL

Division of Bacteriology, National Institute of Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Herts EN6 3QG, England

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SUMMARY

Examination of a selection of serum samples from adults from two regions of England showed that 50% of men in the 16–24 years and over 55 years age groups had high titres of antibody to diphtheria toxin (DT). In contrast, only 11% of women aged 16 to over 55 years had high titres of antibody to DT. All human antisera with high anti-DT titres reacted with a synthetic peptide (SP) corresponding to the amino acids 141–157 of DT fragment A, with sera from men aged 35 to over 55 years showing the highest titres. High antibody titres to fragment A paralleled those to SP in both sexes. Titres of antibody to DT fragment B were highest in individuals with high titres to DT. In sera from both sexes immunoglobulin G_1 was the predominant subclass reactive with all three antigens. However, both IgG_1 and IgG_4 and to a lesser extent IgG_2 and IgG_3 were present in immunoglobulin concentrates.

INTRODUCTION

Diphtheria toxoids as possible candidate vaccines against diphtheria were proposed in the early 1920s by Ramon [1]. The introduction and use of such vaccines in many countries from the 1940s onwards proved effective in controlling the incidence of diphtheria. In England and Wales for example, mortality from this disease decreased from around 80 cases per million in the 1930s to 0·7 million in 1951 [2]. In the United States of America and in the UK infants are usually immunized with a combination of diphtheria toxoid, tetanus toxoid and *Bordetella pertussis* cells (DTP) [3]. A typical immunization schedule for initial vaccination consists of a first dose at approximately 6 weeks of age (1st primary), 4–8 weeks after the first dose (2nd primary), 4–8 weeks after the second dose (3rd primary) and approximately 1 year after the third dose (4th primary). Booster doses are administered at 4–6 years of age before the child enters kindergarten or primary school. Immunity is expected to last throughout life.

* Present address: Seradyn Inc., 1200 S. Madison Avenue, Indianapolis, IN 46225, USA.

Although diphtheria is now a rare disease in the United Kingdom and in many developed countries, recent evidence appears to indicate that under some circumstances this initial course of immunization may be ineffective in preventing sporadic cases of diphtheria among adults [4–6]. According to Galazka and Keja [7], the annual incidence of adult diphtheria in the USSR and Turkey exceeds 1000 and 100 respectively. Furthermore, a report by Christenson and Böttiger [8], indicates the existence of a large pool of susceptible individuals with potential for epidemic infection in Sweden. Similar populations are reported in other countries [7]. Susceptibility to diphtheria in adults appears to be dependent on both the sex and age [7, 8]. The highest risk group (with the lowest frequency of protective levels of diphtheria antibodies) is between 20 and 50 years of age, with women generally having a lower prevalence of immunity than men. In the United States of America the re-immunization of such populations with the adult diphtheria (and tetanus) toxoids every 10 years has been recommended in order to maintain a high level of protection against diphtheria [9].

In addition to improved immunization programmes, the use of novel vaccines that elicit 'long lasting' neutralizing antibody is also important in the prevention and control of the disease. Proteolytic cleavage followed by reduction of diphtheria toxin (DT), generates two fragments; fragment A with ADP-ribosyl transferase activity and fragment B which binds to host cell receptors [10]. Detoxified products from CRM mutants 197 and 45 with mutations in A and B fragments respectively [11–14] may be considered as possible future vaccines. Another approach is the use of synthetic peptides (SP) corresponding to epitopes of the B fragment which elicit neutralizing antibody [15, 16].

As better ways to improve immunity to diphtheria are being pursued, the need for meaningful *in vitro* assays to monitor the humoral antibody response becomes equally important. This is particularly so when the Schick test, which is used today to distinguish the 'immune' from the 'non-immune', cannot be correlated with antibody response [17]. Although the distribution of antibody levels to DT in various populations is well documented [8, 18–20], there is little information on the antibody responses to individual fragments and defined epitopes. Apart from observations on a synthetic oligopeptide of the loop of fragment B (amino acids 188–201) which has been used successfully as a vaccine against lethal DT intoxication in guinea-pigs [15, 16], very little is known about SPs derived from fragment A.

This paper describes a survey carried out on several pools of adult human sera for antibodies to DT, and fragments A and B. We also report, for the first time, the antibody response to a SP which incorporates the ADP-ribosylation site of fragment A. The sera were from men and women of age group 16–55 years, and were collected from two geographic regions in England.

MATERIALS AND METHODS

Antigens

A SP corresponding to the amino acid sequence 141–157 of fragment A (deduced from Kaczorek and co-workers, [21]) was custom synthesized by Cambridge Research Biochemicals (Cambridge, UK). The sequence was Ala-Glu-

Gly-Ser-Ser-Ser-Val-Glu-Tyr-Ile-Asn-Asn-Trp-Glu-Glu-Ala-Lys. Before use, the SP was coupled to either bovine serum albumin (BSA) (Sigma) or keyhole limpet haemocyanin (KLH) (Sigma) as follows. Synthetic peptide (1 mg) was dissolved in 1 ml 0·01 M sodium phosphate buffer, pH 7·4 containing 0·15 M sodium chloride (PBS). To this was added an equal volume of either 3 mg/ml BSA or 2 mg/ml KLH. Glutaraldehyde (EM Grade, Sigma) was then added to a final concentration of 0·04% (v/v) and the reaction mixture was incubated at room temperature for 1 h. Excess aldehyde groups were blocked by addition of 0·5 ml 5 M ethanolamine. The coupled peptides (SP-BSA, SP-KLH) were dialysed against 100 volumes of PBS (three changes) for 24 h at 4 °C before storing at 4 °C with 0·01% (w/v) thiomerisol as preservative.

Purified DT (5 mg/ml protein, 949 Lf/ml) was provided by Dr A. Whitaker (Wellcome Research Laboratories, Beckenham, Kent). For isolation of fragment A, DT solution was concentrated threefold by ultrafiltration (YM30 membrane, Amicon Corp) and 100 mg of DT were used for purification of fragment A according to the method of Drazin and colleagues [22]. Purified fragment A was lyophilized and stored in a desiccator at −20 °C.

For isolation of fragment B, DT (5 mg/ml) was treated with trypsin (1 μg/ml) (Sigma) as for fragment A and samples were subjected to gel electrophoresis as described below. Protein bands were visualized by staining lightly with 0·03% (w/v) Coomassie Brilliant Blue R250 (CBB) (Sigma), and the band corresponding to fragment B was excised. The excised gel was thinly sliced and fragment B was electroeluted using the Isco electrophoretic concentrator model 1750 (Isco Inc., Lincoln, Nebraska) according to manufacturer's instructions. The eluted protein (approximately 600 μg/ml) was stored at 4 °C with 0·01% (w/v) thiomerisol as preservative.

Antisera

One female New Zealand White rabbit was test bled for control serum 1 week before immunization. The animal then received two immunizations, each with two subcutaneous and two intramuscular injections of SP-KLH as follows. The first immunization was carried out with 100 μg of SP-KLH emulsified with Freund's complete adjuvant (Difco). One month later, a booster dose was given with 50 μg of antigen emulsified with Freund's incomplete adjuvant (Difco). Three weeks later, the animal was exsanguinated and the antiserum was harvested and stored at −20 °C.

Human sera from five age groups (16–24, 25–34, 35–44, 45–54 and over 55 years) from both sexes were selected from samples submitted for assessment of diphtheria and tetanus antitoxin status and from a collection provided by Dr P. Morgan-Capner of the Public Health Laboratory, Preston. The latter samples had been collected from healthy adults in the north and south-east of England for an unrelated serological study. Sera were also available from laboratory staff who had been re-vaccinated with diphtheria vaccine for adults. With the exception of the latter, the vaccination history of the serum donors is believed to be typical of the populations from which they were drawn. Samples of commercial immunoglobulin concentrates manufactured from UK serum donations and one batch of human antitetanus immunoglobulin were also examined.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were dissolved in SDS and 2-mercaptoethanol (Sigma) and examined by SDS-PAGE using 12·5% (w/v) acrylamide gels [23]. The gels were stained with 0·05% (w/v) CBB and destained with 5% (v/v) acetic acid in 10% (v/v) methanol.

Immunoblotting

Electrophoretic transfer of proteins from SDS-PAGE gels to nitrocellulose paper was carried out according to the method of Towbin and co-workers [24]. After transfer, the nitrocellulose was blocked with 3% (w/v) BSA in PBS for 1 h at 45 °C, washed three times in PBS containing 0·05% Tween 20 (PBST) and incubated for 2 h at 37 °C with a 1:100 dilution of rabbit antiserum to SP-KLH (also diluted in PBST). After washing with PBST as above, the immunoblot was incubated with a 1:1000 dilution of goat anti-rabbit immunoglobulin (Ig) conjugated to horseradish peroxidase (HRP) (Sigma) in PBST for 45 min at room temperature. After a further washing step, the substrate was added. This was 40 mg 4-chloro-1-naphthol (Sigma) dissolved initially in 20 ml methanol and diluted to 100 ml with PBS. Hydrogen peroxide was added to a final concentration of 1·3 mM immediately before use.

Enzyme-linked immunosorbent assays (ELISAs)

Initial screening of human sera for DT antibodies by ELISA was carried out essentially according to Melville-Smith and Balfour [25]. The volume of reagent used in all assays was 100 μl/well unless otherwise stated. Polystyrene flat bottom microtitre plates (NUNC, Bacteriological grade) were coated with ca. 5 μg/ml DT in 0·1 M sodium bicarbonate/carbonate buffer, pH 9·6 (coating buffer) for 2 h at 4 °C.

Antibody titres (in IU/ml) of unknown sera were determined by comparison with a standard antitoxin with a titre of 1·25 IU/ml. The data were analysed as parallel line assays.

Each age group and sex was further subdivided into two groups by pooling sera with high (0·5–2.0 IU/ml) and low (0·04–0·15 IU/ml) titres to DT. Sera with moderate (0·16–0·49 IU/ml) titres were excluded from further study. Each pool of sera was then examined for antibodies to fragments A, B and SP by ELISA as follows. Polystyrene flat bottom microtitre plates (NUNC, Immunoplate II) were coated with fragment A (2 μg/ml) in coating buffer for 16 h at 4 °C The plates were washed three times with PBST and blocked with 3% (w/v) BSA in PBS for 1 h at 37 °C. After washing, twofold serial dilutions of pooled sera (diluted initially to 1:100) were added and the plates incubated for 2 h at 37 °C The plates were then washed and incubated with a 1:1500 dilution of rabbit anti-human Ig conjugated to HRP (Sigma) for 45 min at 37 °C. After a further washing step, peroxidase activity was assayed with 3,3′, 5,5′ tetramethylbenzidine (TMB) (Sigma) for 15 min. The TMB (10 mg) was dissolved initially in 1 ml dimethyl sulphoxide and added dropwise to 100 ml 0·1 M sodium acetate/citric acid buffer, pH 6·0. Hydrogen peroxide was added to a final concentration of 1·3 mM to the TMB solution just before use. The reaction was stopped by addition of 50 μl per

well of 3N sulphuric acid and the absorbance due to the yellow colour was determined at 450 nm. Antibody titres of pooled sera were determined by comparison to a standard serum. Since the standard serum (at 1·25 IU/ml) was titrated against whole DT, antibody titres to fragments A, B and SP were expressed as IU equivalents/ml. All data were analysed as described before.

Titration of rabbit antiserum to SP-KLH against native DT, fragment A and SP-BSA was carried out as described above but using rabbit antiserum diluted initially to 1:100 and a 1:1000 dilution of goat anti-rabbit-Ig conjugated to HRP. Immunoglobulin concentrates were titrated against DT essentially as described above but using wells coated with DT at 5 μg/ml.

Human IgG subclass types to DT were determined by ELISA as follows. Wells were coated with antigen and blocked with BSA as described above. Then 1:100 dilutions of antibody-containing samples were added as above, washed, and incubated with a 1:1000 dilution of mouse monoclonal antibodies to human IgG_1, IgG_2, IgG_3 and IgG_4 (Sera Lab, Crawley Down, Sussex) for 1 h at 37 °C. The plates were then washed and incubated with a 1:1000 dilution of goat anti-mouse Ig conjugated to HRP (Sigma) for 45 min at 37 °C. Peroxidase activity was assayed with TMB as described earlier.

Estimation of protein

Protein concentrations were estimated by the dye-binding method of Bradford [26].

RESULTS

Antigenic similarity of SP to native antigen

The rabbit antiserum to SP-KLH when examined by ELISA, reacted with SP-BSA as well as native DT and purified fragment A with titres of 1:51200, 1:12800 and 1:51200 respectively. Examination of SP-BSA by SDS-PAGE (Fig. 1, track 3) revealed a broad diffuse band in contrast to a distinct band formed by BSA alone (track 4). The antiserum to SP-KLH reacted with SP-BSA on immunoblots to produce a similar diffuse band (Fig. 2, track 3). Purified DT (Fig. 1, track 2) formed three major bands corresponding to intact toxin (63 kDa), fragment B (39 kDa) and fragment A (24 kDa). Purified fragment A (Fig. 1, track 1) produced a major band of 24 kDa and a few minor bands of lower molecular weight. The anti-SP antiserum when examined by immunoblotting, reacted with fragment A as well as the minor bands (Fig. 2, track 1) and specifically labelled fragment A and intact DT (track 2) with no labelling of fragment B.

Antibody response to DT, fragments A, B and SP

The fragment A and DT preparations used in this study were free of contaminating antigens (Fig. 1, tracks 1, 2). (The minor bands present in fragment A were derived from fragment A.) Fragment B electroeluted from gel slices formed a single diffuse band when re-examined by SDS-PAGE (data not shown).

Results from a survey carried out to determine the distribution of high and low antibody titres to DT in men and women of five age groups are summarized in Table 1. Fifty percent of men and only 11% of women of 16–24 years of age had high levels of antibody to DT, while the corresponding figures for low titres were

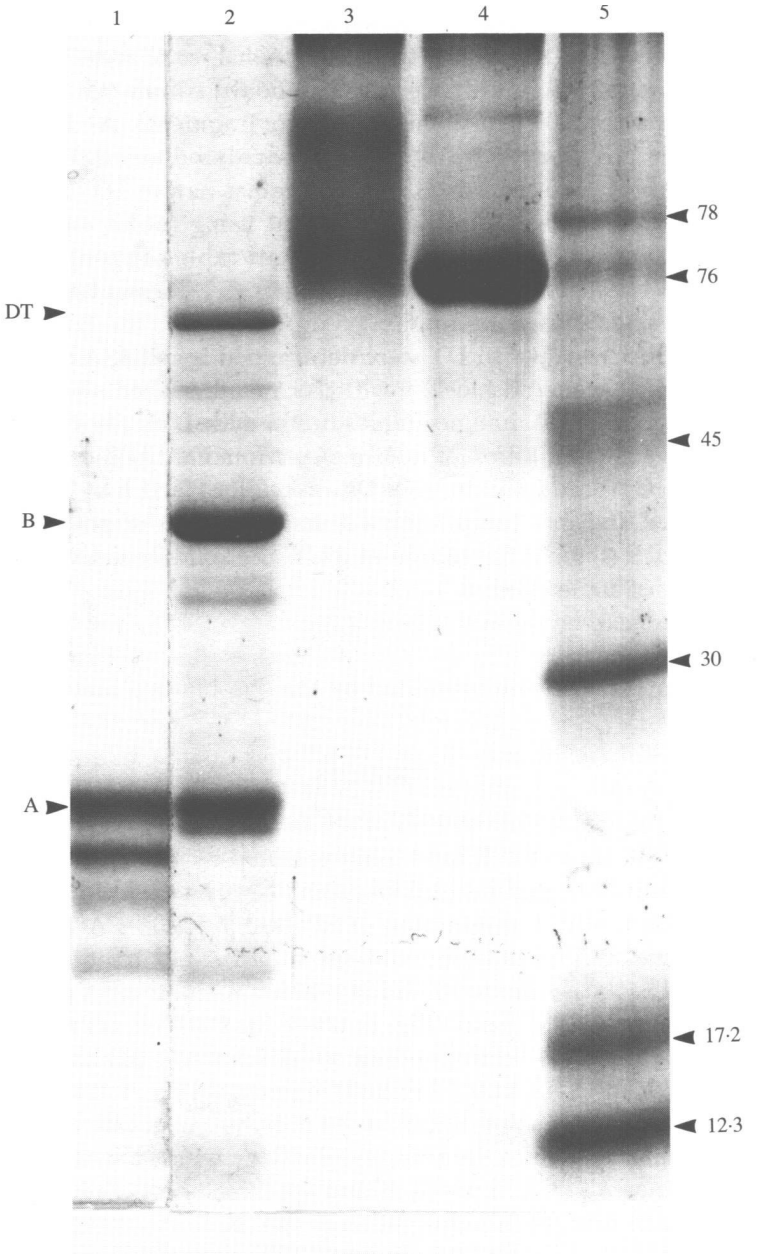

Fig. 1. The SDS-PAGE pattern of purified antigens. Track 1, purified fragment A (2 μg). Track 2, purified DT (10 μg). Track 3, SP-BSA (15 μg). Track 4, BSA (5 μg). Track 5, M_r standards. Positions of intact DT, fragments A and B are shown.

17% and 67% respectively. In contrast, only 13% of men of 25–34 years of age had high levels of antibody while the proportion for women was 19%. Approximately half the samples from both men and women (60% and 52% respectively) of this age group had low antibody titres. In the age group 35–44 years the proportion of men with high antibody titres increased to 43% while for females the corresponding figure was 17%. The proportion of men and women of

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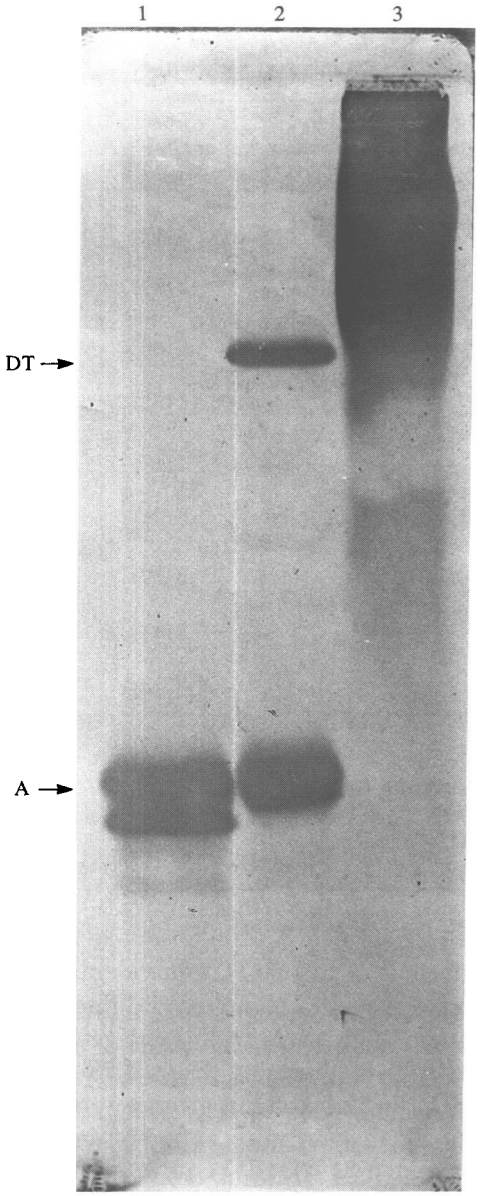

Fig. 2. Immunoblot analysis of rabbit anti-SP antiserum. Track 1, purified fragment A (2 μg). Track 2, purified DT (5 μg). Track 3, SP-BSA (10 μg). Positions of intact DT and fragment A are shown.

this age group having low antibody titres was similar (17 % and 26 % respectively) with the remainder having moderate antibody titres. A significant proportion of men (45 %) and women (38 %) of age group 45–54 years gave high antibody titres, with 27 % of men and 44 % women showing low levels of antibody. Half the men and 14 % of the women over 55 years of age had high levels of antibody while the corresponding figures for low antibody levels were 40 % and 57 % respectively.

The number of pooled sera are shown in Table 1 and antibody titres of pooled sera to individual fragments and SP are summarized in Fig. 3. In samples from

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Table 1. *Antibody response to DT as determined by ELISA*

Sex	Total sera examined	Age group (years)	Number of sera with* High antibody levels†	Number of sera with* Low antibody levels‡	Number of sera pooled	Group designation
Male	18		9 (50)§		5	M1H
		16–24		3 (17)	3	M1L
Female	18		2 (11)		2	F1H
				12 (67)	5	F1L
Male	15		2 (13)		2	M2H
		25–34		9 (60)	6	M2L
Female	21		4 (19)		2	F2H
				11 (52)	7	F2L
Male	23		10 (43)		6	M3H
		35–44		4 (17)	4	M3L
Female	23		4 (17)		3	F3H
				6 (26)	5	F3L
Male	11		5 (45)		4	M4H
		45–54		3 (27)	4	M4L
Female	16		6 (38)		3	F4H
				7 (44)	5	F4L
Male	10		5 (50)		3	M5H
		> 55		4 (40)	3	M5L
Female	24		2 (14)		2	F5H
				8 (57)	4	F5L

* Moderate antibody titres excluded.
† 0·5–2·0 IU/ml.
‡ 0·04–0·15 IU/ml.
§ Numbers in parentheses are percentages of total sera examined.

men, high antibody titres to fragment A were observed in groups M2H, M4H and M5H with titres ranging from 2·70 to 2·90 IU equivalents/ml. Comparison of sera with low and high antibody levels to DT revealed differences in anti-A fragment titres of 1·5–5 fold. In women, high titres to this antigen were observed in groups F1H, F3H and F5H with titres ranging from 1·78–2·27 IU equivalents/ml. Differences in titres between the high and low groups were approximately 2–4 fold.

High antibody titres to SP in men were apparent in groups M3H, M4H and M5H with values ranging from 1·81–2·04 IU equivalents/ml. Differences in titres were 1·5–3 fold. Female groups with high antibody titres to SP were F2H, F3H, F4H and F5H with values ranging from 1·21–1·83 IU equivalents/ml. Differences in anti-SP titres between the high and low groups for all five age groups of women were 1·5–2·5 fold. In general, the difference in anti-SP antibody titres in people with high levels of antibody to DT relative to those with low titres, paralleled the values for antibody to fragment A. This was apparent in all groups except F1H and M2H where the antibody level to fragment A was nearly 2-fold higher than that for SP.

Pooled sera from men that showed high titres to fragment B were M2H, M4H and M5H with titres ranging from 2·51–2·84 IU equivalents/ml. Overall differences in anti-B fragment titres were 2–9 fold. In women, the corresponding groups were F1H, F2H, F3H and F5H with titres ranging from 1·98–2·34 IU equivalents/ml.

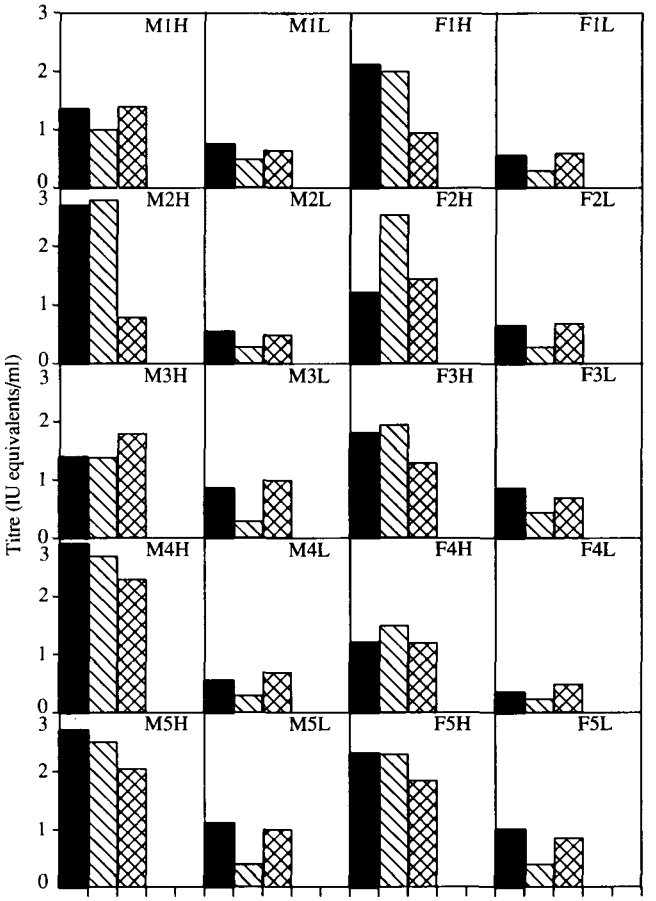

Fig. 3. Antibody titres of pooled sera to fragments A, B and SP as determined by ELISA. ■, anti-fragment A; ▨, anti-fragment B; ⊠, anti-SP.

The differences in anti-B fragment titres observed were 4–8 fold. The highest anti-B fragment titres relative to those against fragment A were observed in subjects who had recently received booster doses of diphtheria vaccine for adults.

Antibodies of the IgG_1 subclass were present against all three antigens in all the pooled sera examined. In addition, antibodies of the IgG_4 subclass against fragments A and B were detectable in M2H, M3H and F3H.

Examination of 20 human immunoglobulin concentrates and one immunoglobulin preparation intended for tetanus prophylaxis showed that all contained high antibody activity to DT (mean $\pm$S.D. = $2{\cdot}70 \pm 0{\cdot}34$ IU/ml). In all these most of the antibody activity was associated with the IgG_1 and IgG_4 subclasses but seven preparations also showed low levels of activity in the IgG_2 and IgG_3 subclasses.

DISCUSSION

The results of examination of the human serum pools were consistent with previous observations on the persistence of immunity to diphtheria in other developed countries. A moderate proportion of men (50%) 16–24 years of age showed high levels of antibody to DT probably indicating that immunity acquired

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from vaccination at childhood persisted in males of this age group. A similar percentage of men over 55 years of age also showed high levels of antibody to DT. This could have resulted from either exposure to natural infection at some time or from re-immunization later in life, most likely during military service. The observation that women of all age groups and men 25–54 years of age, in general, had low levels of antibody to DT, was consistent with previous reports from other countries [8].

The close parallel between antibody titres to fragments A and B and those to DT was not unexpected. However, the relatively greater increase in titres to fragment B noted in sera from recently re-vaccinated subjects could be of particular significance as experimental evidence has shown that antibodies to this part of the DT structure are protective whereas those directed against the A fragment are not [3]. The demonstration of substantial antibody activity towards DT in immunoglobulin concentrates could be of clinical relevance as, in contrast with tetanus, no specific human anti-diphtheria immunoglobulin preparations are available. The observation that most of the antibody activity to DT detected in serum was associated with the IgG_1 and to a much smaller extent to the IgG_4 subclass, with activity in IgG_2 and IgG_3 detectable only in immunoglobulin concentrates, closely paralleled the findings of Seppalä and colleagues [27] for antibodies to tetanus toxoid. The reason for the dominance of IgG_1 in the human antibody response to protein toxins is unknown.

There is conflicting information in the literature as to the exact amino acid sequence of fragment A and the precise location of the catalytic site with ADP-ribosyl transferase activity. According to the earlier work of Kandel and colleagues [28], at least one tryptophan residue of fragment A was involved in binding to NAD, whereas Giannini and co-workers [13] have suggested that glycine (amino acid 52) was the probable catalytic site. Since subsequent work of Tweten, Barbieri and Collier [29] has shown that glutamic acid (amino acid 148) was the catalytic site with ADP-ribosyl transferase activity, the amino acid sequence 141 to 157 (with glutamic acid at position 148) was chosen as the sequence for the SP used in this study. The complete sequence of SP was deduced from that reported for fragment A by Kaczorek and colleagues [21] even though this particular sequence was inconsistent with the data of DeLange, Williams and Collier [30]. Rabbit antibodies to conjugated SP recognized the native intact DT as well as purified fragment A suggesting that the peptide was antigenically similar to the native epitope(s) of the intact molecule.

Rabbit antisera to diphtheria toxoid however, failed to react with conjugated SP (unpublished data) whereas human sera were reactive suggesting that the former may be directed primarily against tertiary epitopes of the native antigen. Since SP was antigenically similar to fragment A, as expected, increases in anti-SP antibody titres (in people with high levels of antibody to DT), paralleled those of antibody to fragment A. This was apparent in all groups except women 16–24 years and men 25–34 years of age. Comparison of sera with low and high antibody to DT, also revealed that elevation of antibody to fragment B was the most pronounced and may have played a major role in the overall elevation of antibody titres to DT in some populations. Furthermore, immunization of individuals with low antibody titres to DT with the adult diphtheria vaccine has been shown to

cause a 3–4 fold increase in anti-B fragment antibody of IgG_1 subclass with no significant increase in anti-A fragment antibody (unpublished data). These observations however, do not support those of Triebel and colleagues [31] who have reported that 80% of the immune response was directed against epitopes on fragment A with minimal response to fragment B.

Since antibody to fragment B, particularly against the loop is known to neutralize intact DT [3, 15, 16], it is important that all future vaccines elicit high levels of such antibody in order to confer protection against diphtheria. Monitoring of antibodies to individual fragments or epitopes of fragments should therefore be considered in future, since such data would be more meaningful than those based on whole toxin in assessing the precise immune status of different populations.

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