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Opsonic human antibodies from an endemic population specific for a conserved epitope on the M protein of group A streptococci

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SUMMARY

This study demonstrates the presence of epitope-specific opsonic human antibodies in a population living in an area endemic for group A streptococci (GAS) infection. Antibodies recognizing a conserved C-terminal region epitope (p145, sequence in single letter amino acids: LRRDLDAS-REAKKQVEKALE) of the M protein of GAS were isolated from human patients by affinity chromatography and were shown to be of the immunoglobulin G1 (IgG1) and IgG3 subclasses. These antibodies could reduce the number of colonies of serotype 5 GAS in an *in vitro* opsonization assay by 71–92%, compared with an equal amount of IgG from control adult donors living in non-endemic areas and without antibodies to p145. Addition of the peptide, p145, completely inhibited this opsonization. Indirect immunofluorescence showed that p145-specific antibodies were capable of binding to the surface of M5 GAS whereas control IgG did not. Using chimeric peptides, which contain overlapping segments of p145, each 12 amino acids in length, inserted into a known helical peptide derived from the DNA binding protein of yeast, GCN4, we have been able to further define two minimal regions within p145, referred to as pJ2 and pJ7. These peptides, pJ2 and pJ7, were able to inhibit opsonization by p145 specific antibodies. Finally, we have observed an association between the age-related development of immunity to GAS and the acquisition of antibodies to the conserved epitope, p145, raising the possibility of using this epitope as a target in a prophylactic vaccine administered during early childhood.

INTRODUCTION

Group A streptococci (GAS) can cause skin and throat infections in humans as well as post-infectious sequelae including acute rheumatic fever (ARF). ARF predominantly affects people living in overcrowded areas with a low standard of health. Despite the fact that streptococcal infection can be successfully treated with antibiotics, the incidence of ARF has increased in some tropical countries with rates at times over 100/100 000 per year.[1] In Australian Aboriginal populations the prevalence of established rheumatic heart disease (RHD) can be as high as 30/1000.[2,3]

The surface M protein is considered the major virulence factor of GAS. Antibodies directed to the highly antigenically variable N-terminal region of the M protein, however, can opsonize bacteria of the homologous serotype for uptake by neutrophils. Development of a vaccine to GAS, and thus to RF, has been hampered by the diversity of serological M types. One approach for a GAS vaccine has been to incorporate several distinct opsonic N-terminal epitopes;[4–6] however, this may not be practical given the large number of serological M types (in excess of 80[7]). Another approach has been to investigate the highly conserved C-terminal region of the M protein. Data from others and ourselves[8–13] suggest that the conserved region may induce immunological protection against GAS infection. Antibodies raised in mice to a conserved 20-mer peptide (referred to as p145) were able to opsonize reference strains of GAS as well as GAS isolates taken from Aboriginal and Thai patients with RF.[12,13] The M protein is known to form a coiled coil α-helix and recently we have shown that p145 has α-helical potential.[14] By inserting overlapping segments of p145, each 12 amino acids in length, into a known helical peptide derived from the DNA binding protein of yeast, GCN4 (thus creating 'chimeric' peptides), we have been able to map minimal B-cell epitopes within p145. Sera from one group of Aboriginal subjects with a high titre of antibodies to p145 were shown to recognize a chimeric peptide referred to as J2, thereby mapping the minimal B-cell epitope to the N-terminal region (residues 2–13) of p145.[14]

To gain insight into the development of immunity to GAS, we have how determined whether purified human anti-p145-specific antibodies can directly opsonize the bacteria and can

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bind to the surface of the bacteria; we have investigated whether defined conformational epitopes within p145 are the targets of opsonic antibodies, and we have studied the acquisition of antibodies to p145 and its epitopes using two age-stratified populations from streptococcal-endemic areas of Australia and Papua New Guinea.

MATERIALS AND METHODS

Patients

Sera was obtained from a total of 129 Australian Aborigines from the Northern Territory with established rheumatic heart disease (RHD) ($n = 46$), acute rheumatic fever (ARF) with or without carditis ($n = 20$), other non-rheumatic heart disease ($n = 7$), other streptococcal disease ($n = 5$), and control subjects with no history of ARF and no clinical signs of RHD ($n = 51$). All subjects were assessed by physicians, and patients with ARF met the revised Jones criteria for diagnosis.[15] The majority of patients with RHD were receiving penicillin prophylaxis. Age-stratified sera were also obtained from 111 normal Papua New Guinean (PNG) subjects.

Peptides

Synthetic peptides were made as described[16] and were highly purified by high performance liquid chromatography (HPLC). The peptide of interest, p145, has the 20 amino acid sequence Leu-Arg-Arg-Asp-Leu-Asp-Ala-Ser-Arg-Glu-Ala-Lys-Lys-Gln-Val-Glu-Lys-Ala-Leu-Glu. The sequences of the chimeric J peptides, J1–J9, are given in Table 1. A non-specific peptide from an unrelated *Schistosoma* antigen with the amino acid sequence Glu-Gly-Lys-Val-Ser-Thr-Leu-Pro-Leu-Asp-Ile-Gln-Ile-Ile-Ala-Ala-Thr-Met-Ser-Lys was also used.

Detection of antibodies

An enzyme linked immunosorbent assay (ELISA) was used to measure human serum antibodies to the peptides as previously described[13] with the addition that standard curves of optical density versus known concentrations of human IgG were used to calculate antibody concentration, as described.[17] Subclass immunoglobulin ELISAs were performed, essentially as described,[12] with the exceptions that the 100 μl of biotin conjugated anti-human IgG1, IgG2, IgG3, and IgG4 (Sigma, Sydney, Australia) diluted 1:200, 1:300, 1:100 and 1:100 respectively were added to the primary antibody on the plate and incubated overnight at 4°. Plates were washed three times and 100 μl of Extravidin (Sigma) conjugated to alkaline phosphatase substrate (100 μl) (Sigma) was added according to the manufacturers' instructions and incubated at 37° for 2 hr or 4° overnight. Absorbance was read at 405 nm and significance was determined as a value at a dilution of 1:100 of sera that was greater than the mean of the blank by three standard deviations.

Table 1. List of synthetic peptides

145	**LRRDLDASREAKKQVEKALE**
Jcon	DKVKQAEDKVKQLEDKVEELQDKVKQLE
J1	QLEDKVKQ**LRRDLDASREAK**EELQDKVK
J2	LEDKVKQA**RRDLDASREAKK**ELQDKVKQ
J3	EDKVKQAE**RDLDASREAKKQ**LQDKVKQL
J4	DKVKQEAD**DLDASREAKKQV**QDKVKQLE
J5	KVKQAEDK**LDASREAKKQVED**KVKQLED
J6	VKQEADKV**DASREAKKQVEK**KVKQLEDK
J7	KQAEDKVK**ASREAKKQVEKA**VKQLEDKV
J8	QAEDKVKQ**SREAKKQVEKAL**KQLEDKVQ
J9	AEDKVKQL**REAKKQVEKALE**QLEDKVQL

Purification of antibodies

Human immunoglobulins were purified from whole serum by Protein A Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden).[18] Antibodies were eluted with 0·1 M citric acid, pH range 6·5–3 to elute all IgG isotypes.[18] To isolate p145 specific antibodies, an immunoabsorbent was prepared by synthesizing p145 on Novasyn TG resin (Calbiochem-Novabiochem, Cysatstrasse, Switzerland) without first adding a linker to the support. Fmoc chemistry was used throughout the assembly and the side chain protecting groups were removed at the end of the synthesis using reagent B (trifluoroacetic acid (TFA), phenol, water, triisopropylsilane in the ratio 88:5:5:2) at room temperature for 4 hr. Absence of a linker between the support and peptide results in a deprotected peptide covalently attached to the solid phase support through its carboxyl terminus. The support is based on polyethylene glycol and its hydrophilic nature lends itself well to use with aqueous solutions including the absorption of antibodies from sera. A similar approach has been described.[19] Protein A purified immunoglobulins were passed over the column, washed with phosphate-buffered saline (PBS), pH 8·0 and eluted with 6 M guanidine HCl, pH 3·0. For buffer exchange to PBS, pH 7·4, the eluate was passed over a disposable PD-10 Sephadex G-25 M column according to manufacturers' instructions (Pharmacia) and concentrated to one-tenth of the original sera volume using a centricon-100 unit (Amicon, Beverly, MA).

Bactericidal assay

Purified human antibodies and p145-specific immunoglobulins were assayed for their ability to opsonize GAS M type 5 as described.[12–14,20] Briefly, the bacteria were grown overnight at 37° in 5 ml Todd–Hewitt broth (THB), and then diluted 10^{-5} in saline. Fifty microlitres of the bacteria dilution was mixed with 50 μl of purified IgG and 400 μl of non opsonic heparinized donor blood. The mixture was incubated end over end at 37° for 3 hr and 50 μl from each tube was plated out in duplicate on blood agar pour plates, incubated overnight and the number of colonies on each plate counted.

Peptide inhibition assays were performed as described,[13,14,21] 100 μg of peptide was added to purified IgG 30 min prior to the addition of bacteria and heparinized blood as described above. Percentage blocking was determined by comparison of the number of colonies growing in the presence of specific peptide to number of colonies growing in the presence of a non-specific peptide.

Indirect immunofluorescence

Bacteria were cultured overnight in 5 ml of THB at 37°, centrifuged, washed twice in PBS and resuspended in PBS to an $OD_{540} = 0{\cdot}1$. Fifty microlitres of the bacterial dilution was dropped onto duplicate slides, air dried and fixed for 10 min in 3% paraformaldehyde (Sigma) in PBS. Slides were washed twice in PBS and cells were blocked with 50 μl of 5% skim milk in PBS for 30 min in a humidified chamber. Antibody samples

diluted 1 : 100 in 0·5% skim milk in PBS were added to each slide and incubated at 4° overnight in a humidified chamber. Slides were washed three times in PBS and 50 μl of sheep anti-human IgG (Calbiochem) diluted 1 : 10 were added to each slide and incubated at room temperature for 2 hr. Slides were washed again and 50 μl of fluorescein isothiocyanate (FITC)-conjugated anti-sheep IgG (Silenus, Hawthorn, Australia), diluted 1 : 20, were incubated for 30 min in a humidified chamber. Slides were washed in PBS before cells were mounted in mounting media containing an anti-fading agent (PBS/glycerol; 9 : 1, 0·1% *p*-phenylenediamine [Sigma]) and observed on an Olympus BA2 microscope using a narrow band blue filter of 488 nm at ×1000 magnification.

Statistical analysis

Geometric means and standard deviations were calculated using standard formulas. Comparison between groups of patients was performed using the one tailed Student's *t*-test for unpaired means. Statistical significance was taken as $P < 0{\cdot}05$.

RESULTS

Prevalence of antibodies to the conserved epitope in age-stratified populations

Sera were obtained from Australian Aborigines from highly endemic areas and examined by ELISA for the presence of antibodies to the conserved peptide, p145. Aborigines in all disease categories [RHD, controls, other heart disease (OHD), ARF, and other streptococcal diseases] had detectable levels of serum antibodies to p145. No significant difference in the mean anti-p145 serum antibody levels was obtained between any of the patient groups ($P > 0{\cdot}05$) (Fig. 1).

The sera of 129 Aborigines and 111 Papua New Guineans were grouped into three age groups: 24 and 34 children (aged 1–10 years), 29 and 33 teenagers (11–19 years) and 76 and 44 adults (20+ years), respectively. Serum antibody concentration to p145 in the three age groups were examined by ELISA. Among the sera from children, teenager and adult Aborigines, all contained measurable quantities of anti-p145 IgG antibodies (ranging from 0·005–3·2, 0·1–11·2 and 0·1–12·8 μg/ml sera respectively) (Fig. 2a). Detectable levels of anti-p145 IgG were present in 79% of children, 88% of teenagers and 97% of

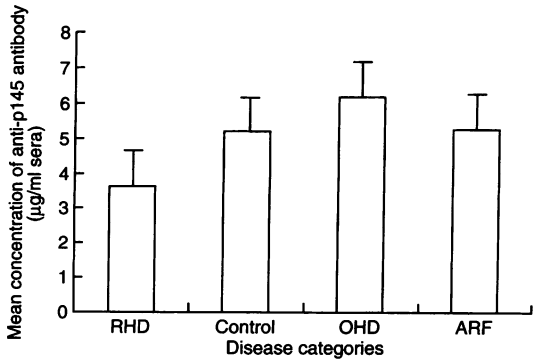

Figure 1. Mean serum antibody concentration against p145 in Aboriginal patient groups.

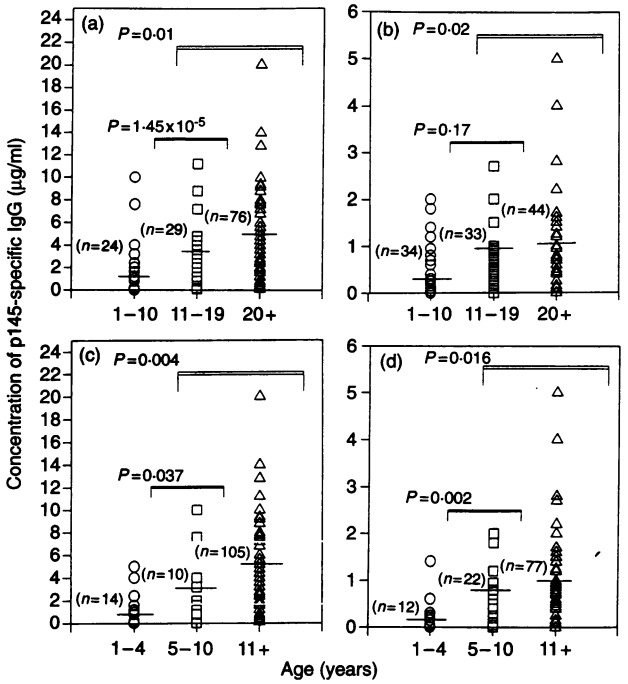

Figure 2. Serum immunoglobulin response to p145 grouped by age. (a) Aboriginal subjects, (b) PNG subjects, (c) and (d) Aboriginal and PNG children respectively shown in Fig. 2a and b, split into the age groups 1–4 and 5–10. Horizontal bars represent mean anti-p145 IgG concentration (μg/ml sera). Each data point may represent more than one set of measurements.

PNG adults surveyed (range, 0–2, 0–0·95, 0–2·5 μg/ml sera respectively) (Fig. 2b). In all three age groups, serum anti-p145-specific IgG concentrations were significantly higher in Aborigines than PNG subjects ($P < 0{\cdot}05$). Both Aboriginal and PNG teenagers and adults had a significantly higher concentration of serum anti-p145-specific antibodies than children ($P < 0{\cdot}01$). Aboriginal adults also had a higher concentration of anti-p145-specific antibodies than the average teenage group investigated ($P < 0{\cdot}05$); however, no significant difference in the mean anti-p145 IgG values was found between the teenagers and adults in the PNG population. The age-related differences in antibody levels was further evident when the age group 1–10 years was divided into the age groups 1–4 years and 5–10 years. Both Aboriginal and PNG children aged 1–4 years had significantly lower levels of antibody to p145 compared to teenagers and adults ($P < 0{\cdot}005$), in contrast to the age group 5–10 years which showed no significant difference to the levels present in teenagers and adults (Fig. 2(c) and (d), respectively).

Mapping the minimal epitope within peptide 145: an age-stratified examination

We have shown by circular dichroism that p145 has α-helical potential.[14] Because of this, minimal epitopes were mapped by designing chimeric peptides constructed to consist of portions of the p145 peptide sequence flanked by sequences from a peptide known to form an α-helical coiled coil, GCN4 (the DNA binding protein of yeast). The chimeric peptides were

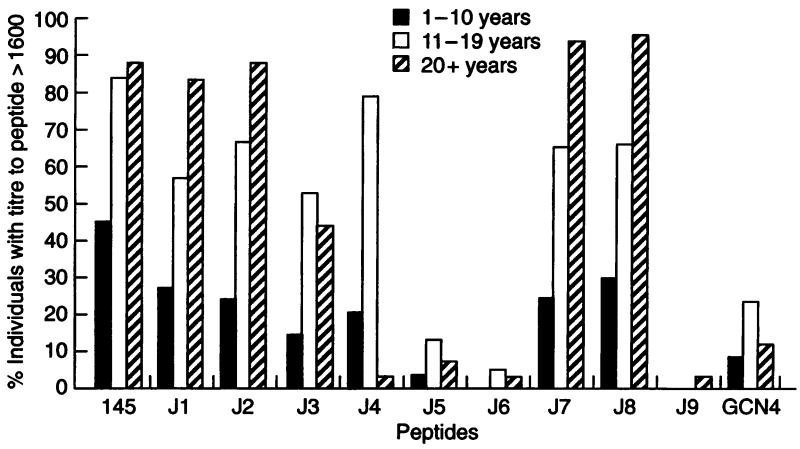

Figure 3. Percentage of Aboriginal subjects, grouped by age, with an ELISA titre > 1600 against p145 and the chimeric peptides J1–J9.

constructed such that the periodicity of hydrophobic residues, consistent with an α-helical coil, was maintained.[14] The chimeric peptides J1 to J9 (Table 1) were used to map the minimal epitope to p145 in the age stratified sera from Aboriginal subjects. We chose 24 Aboriginal subjects aged 1–10 years (titre to p145 ranged from 100 to 12 800), 22 subjects aged 11–19 years (titre 800–12 800) and 23 subjects aged 20+ years (titre 200–12 800) and tested these sera in an ELISA against the chimeric peptides J1–J9. Amongst the age group 20+ years, at least 80% of individuals recognized peptides J1, J2, J7 and J8, followed less commonly by peptide J3, with an antibody titre greater than 3200 (Fig. 3). The recognition of these peptides clearly increased with age with the exception of peptide J4.

Affinity purification and opsonization with serum antibodies specific to peptide 145

In previous studies we have shown that addition of p145 to protein A purified human IgG containing antibodies to p145 was able to block opsonization of M5 GAS,[14] suggesting that human antibodies specific for p145 could opsonize GAS. To confirm this biological activity, we affinity purified antibodies to p145. Total IgG was purified from the sera of three aboriginal patients using a protein A column: an endemic control individual (P17); a patient with non-streptococcal induced heart disease (P101); and a RHD patient (P105). All had high titre antibodies to p145 (> 12 800). IgG was also purified from the sera of two caucasian donors (C1 and C2) with low titre antibodies to p145 (Table 2) and without opsonizing activity (data not shown). Peptide 145-specific antibodies from the three Aboriginal donors were then affinity purified (see Materials and Methods) and assayed by ELISA for their titre to p145, and to peptides J2 and J7 (Table 2). To confirm that p145-specific antibodies were specifically eluted from the affinity column, the antibody titre to tetanus toxoid was determined prior to affinity purification and following elution. None of the affinity-purified immunoglobulins had significant antibody levels to tetanus toxoid following purification as determined by ELISA (Table 2), although the titres prior to purification were high (range: 6400–26 500). The human p145-specific antibodies from the three Aboriginal donors were further characterized for their IgG subclass. All the affinity-purified 145 antibodies were either IgG1 or IgG3 type (Table 2) which are the subclasses of immunoglobulin regarded as most efficient in activation of the classical complement pathway.[22]

Opsonization assays of serotype 5 GAS were then performed with affinity-purified and control immunoglobulins. Control immunoglobulins were from adult donors not living in streptococcal-endemic areas and were prepared as described above. The titres of total IgG for C1 and C2 were determined by ELISA (6 784 000 and 169 600). The immunoglobulin in both samples were then adjusted by serial dilution to a titre of 3200, equal to the total IgG present in the affinity-purified antibody samples. Comparison of the mean number of colony forming units (CFU) in the presence of specific or control antibodies revealed that anti-p145 antibodies could specifically reduce the number of colonies by between 71 and 92% (Table 3). Control immunoglobulin did not bind p145 and was also shown not to contain antibodies specific for the aminoterminus of serotype 5 M protein — an epitope that we have shown is the target of opsonic antibodies.[14]

p145 and the chimeric peptides J2 and J7 inhibit opsonization of GAS mediated by affinity purified p145-specific antibody

We have shown that the Aboriginal population investigated with high titre antibody to p145 could recognize the chimeric peptides J2 and J7 (Fig. 3). Purified p145-specific antibodies from donor sera could also capture the chimeric peptides J2 and J7 in an ELISA assay (Table 2). We then determined whether these peptides could inhibit the opsonization of GAS mediated by affinity purified p145-specific antibodies. Pooled control immunoglobulin and p145-affinity purified immunoglobulin from three donors were tested in opsonization assays. In the presence of a non-specific schistosome peptide, 7405 colonies grew when pooled immunoglobulin (without antibodies to p145) was used (Table 4). In the presence of individual p145-affinity purified immunoglobulin with the non-specific peptide, between 540 and 3450 colonies grew. Addition of p145, to the p145-purified antibodies from the three Aboriginal donors could completely reverse the inhibition, with between 7360 and 11 400 colonies growing (Table 4).

Table 2. ELISA binding titre of human serum IgG to peptide 145, J2, J7 and tetanus toxoid following protein A and 145-affinity column purification

	ELISA titre against:					
Sera	p145	pJ2	pJ7	TT	Total	IgG
Pre-protein A column sera						
P17	12800	6400	3200	12800		
P101	12800	800	12800	26500		
P105	12800	3200	12800	6400		
C1	400	<200	<200	>26500		
C2	800	800	800	>26500		
Protein A purified*						
P17	12800	6400	3200	12800		
P101	12800	800	12800	26500		
P105	12800	3200	12800	6400		
C1	400	<200	<200	>26500	6784000	
C2	800	400	400	>26500	1696000	
Affinity-purified to p145†						
P17	6000	3200	nt	<200	6000	IgG1/IgG
P101	3200	200	3200	<200	3200	IgG1/IgG
P105	3200	1600	3200	<200	3200	IgG1/IgG

* Protein A purified antibodies were concentrated to one-fifth of the original volume of serum.

† Affinity-purified antibodies were concentrated to one-tenth of the original volume of serum.

nt, not tested.

Thus, while the affinity-purified immunoglobulins from these patients alone, and at a titre equivalent to that found in their sera, are unable to completely kill the bacteria, the killing can be completely reversed by the addition of p145, confirming that human p145-specific antibodies can opsonize GAS. This confirms our previous data showing that p145 can inhibit opsonization mediated by serum or protein A purified human immunoglobulin containing p145-specific antibodies.[13] Similarly, both J2 and J7 could completely inhibit opsonization (Table 4). In one case, P17, we noticed that the number of colonies grown in the presence of pJ2 far exceeded the number of colonies that grew in the presence of control immunoglobulin. With the same peptide, increased colony growth (but not as excessive) was found with the two other subjects. The reason for this enhancement is not known, but the addition of these peptides or p145 to the two Caucasian control immunoglobulins (C1 and C2) has no effect on opsonization.

Table 3. Bactericidal effect against M5 GAS of p145-affinity purified human sera

Affinity-purified antibody (titre to p145)	Mean CFU*	Mean CFU pooled Caucasian IgG	% kill
P101 (3200)	540	7000	92%
P105 (3200)	2040	7000	71%

* CFU, Mean colony count from 2 plates × dilution factor. M5 GAS inoculum size = 36.

Binding of affinity-purified p145-specific antibody to M5 GAS

Using indirect immunofluorescence we could show that p145 affinity-purified antibodies bound to the surface of M5 GAS. Affinity-purified p145-specific antibodies were pooled from two donors, P101 and P105 (final p145 titre, 3200) and immunofluorescence staining with these antibodies showed strong fluorescence on the surface of the bacteria (Fig. 4a). Addition of pooled control immunoglobulin (described above), with a final IgG titre of 3200, gave only a weak background fluorescence (Fig. 4b) compared with the staining by anti-p145 specific antibodies. These data show that the epitope recognized by anti-p145-specific antibodies is expressed on the surface of M5 GAS.

DISCUSSION

This study directly demonstrates that antibodies to an epitope from the conserved C-terminal region of the M protein, p145 can kill streptococci in an *in vitro* bactericidal assay, thus arguing for the importance of the epitope for immunity to GAS and its potential relevance for use in a vaccine against GAS and rheumatic fever. Immunofluorescence assays were able to confirm that the epitope recognized by these antibodies is expressed on the surface of GAS. We have previously shown that p145 contains an immunodominant conformational

Table 4. Blocking of opsonization by p145, pJ2, pJ7 of affinity purified p145 human antibodies

Donor (Titre to p145)	Mean CFU*				
	Control non-opsonic IgG + non-specific peptide	p145 affinity-purified IgG + non-specific peptide	p145 affinity-purified IgG + p145 (% blocking)	p145 affinity-purified IgG + pJ2 (% blocking)	145 affinity-purified IgG + pJ7 (% blocking)
P17† (6400)	7405	3450	11 400 (154%)	42 500 (573%)	nt
P101‡ (3200)	7405	540	7360 (99%)	13 800 (186%)	6920 (93%)
P105‡ (3200)	7405	2040	9400 (127%)	10 520 (142%)	6200 (84%)

*CFU, Mean colony count from 2 plates × dilution factor. % blocking = percentage inhibition of bactericidal effect by addition of specific peptide calculated against the mean CFU of control non-opsonic IgG in the presence of a non-specific peptide.

†M5 GAS inoculum size = 73.

‡M5 GAS inoculum size = 36.

epitope within its sequence and, in a different population, identified amino acids 2–13 of p145, as defined by the chimeric peptide J2 as the region expressing the epitope.[12] In the current study, we have demonstrated that in addition to J2, human anti-p145 antibodies can also recognize the chimeric peptides J7 and J8. The peptide inhibition assay performed on affinity-purified p145-specific immunoglobulins shows that opsonization by these antibodies can be blocked by J2 and J7. Interestingly, some of the current population sampled were from different Aboriginal communities, including an island community, to those previously investigated. We are currently typing the human leucocyte antigen (HLA) alleles of the different individuals to see whether subtle differences in epitope recognition may be genetically determined.

It is well documented that children up to the age of 10 years in endemic areas have high incidence rates of streptococcal skin and respiratory infections, which decrease rapidly with increasing age.[23] We have shown that both teenage and adult Aboriginal and PNG subjects living in highly endemic areas for GAS have significantly higher levels of serum antibodies to the conserved region epitope than children, particularly those aged up to 4 years. These observations are comparable to previous studies which found that adult sera have strong antibody responses to the conserved region of the M6 protein.[24] Our data provide evidence of an association of an age-related development of immunity to GAS with acquisition of antibodies to a conserved epitope. The acquisition of these antibodies may explain, in part, the reduced incidences of GAS infections and sequelae in adults. Immunization early in childhood with the epitope may induce accelerated immunity to many GAS serotypes. However, a potential risk associated with immunizing with subunits of the M protein is that such immunization may actually promote disease. While the pathogenesis of RF is not known, there is accumulating evidence that it is an autoimmune disease. We have previously shown that the production of heart-specific antibodies following immunization of mice with p145 is minimal.[12] Furthermore, up to 95% of Aboriginal and Thai subjects have high titre antibodies to this peptide and in the vast majority of such individuals there is no evidence of RF/RHD. Other data, however, has shown that the

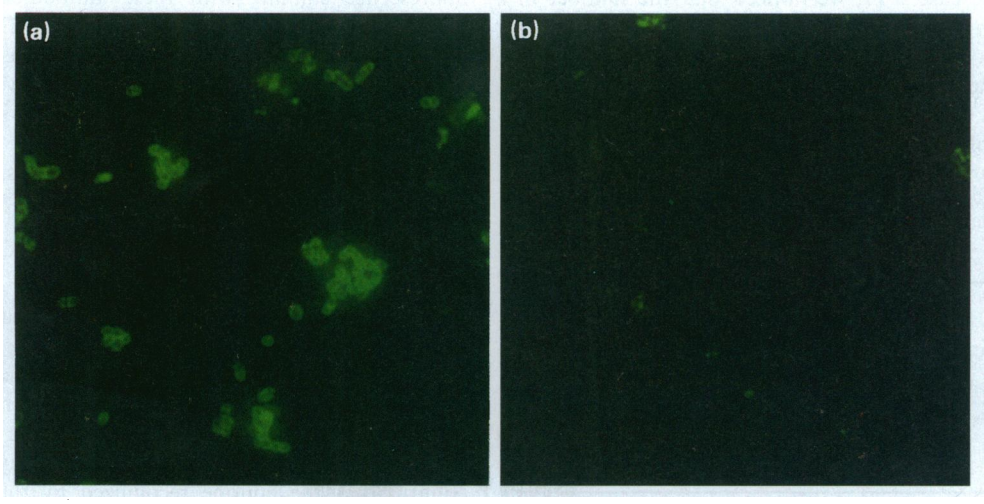

Figure 4. Photomicrographs of immunofluorescent stained M5 GAS (magnification ×1000). (a) M5 GAS exposed to affinity purified p145 specific antibody. (b) M5 GAS exposed to non-opsonic control antibody.

cardiac cellular infiltrates associated with RF are composed predominantly of T cells,[25] and T cells specific for p145 can react with heart tissue and cardiac myosin.[26] Again, though, such responses can be found in normal individuals without any evidence of heart disease. Even though there has not been any association shown between RF/RHD and immune responses to p145, it is not unreasonable to seek as a vaccine candidate a subunit of the M protein which contains the minimal amount of streptococcal sequence. The approach outlined in this study defines possible minimal candidates. The overall strategy is thus similar to that used in diseases such as malaria, where immune responses may be predominantly responsible for pathology, but where non-pathogenic, protective immune responses can be defined.[27]

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