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Serum Antibody Responses of Divers to Waterborne Pathogens

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To assess the significance of exposure of divers to waterborne pathogens, specific immunoglobulin G serum antibody responses to *Pseudomonas* and *Aeromonas* isolates recovered from dive sites and from the respiratory tracts of nine experienced divers and seven diving trainees working in the Chesapeake Bay area over a 6- to 18-month period were measured. A significant increase in the frequency of isolation of these organisms from respiratory surfaces of both groups of divers after each dive was noted, with the divers' ears being the predominant recovery site (48%; $P < 10^{-8}$, chi-square). The acute serum responses of the majority of experienced divers (83%) showed evidence of preexisting antibody to these potential pathogens, whereas the acute serum responses of only 32% of naive divers showed such evidence ($P < 10^{-8}$, chi-square). Six months into their training, the rate of seroresponse of the trainees to organisms recovered after their first dives increased to 61% ($P = 0.003$, chi-square), suggesting that repeated exposure is necessary for generation of a specific systemic immunologic response. The rate of acquisition of a new seroresponse to recovered organisms was approximately 12% per dive for both groups of divers, suggesting that there is continuous exposure to, and infection with, new strains present in the water during dives. These data suggest that, in cases in which systemic antibody is important for protection, there are various levels of susceptibility to waterborne potential pathogens in both experienced and inexperienced divers.

The development of underwater breathing apparatus, coupled with increased recreational and industrial use of fresh and salt water, has exposed humans to new environmental dangers. One noteworthy potential hazard is that associated with infectious agents (3, 10, 15). A number of infectious pathogens, occurring either naturally or as the result of pollution, are present in coastal waters throughout the world (7, 12). Some of these pathogens, most notably *Pseudomonas* and *Aeromonas* spp., have been identified as the causes of skin, mucous membrane, and, occasionally, systemic infections in swimmers and divers (8, 11, 13).

Initial studies, carried out in our laboratory and aimed at defining health risks of divers, showed that potential waterborne pathogens, such as the *Aeromonas* spp., can be recovered from diving equipment, skin, and mucosal sites of the majority of previously uncolonized divers 30 min after a dive (5). Since the first steps in the pathogenesis of infections are adherence and colonization, these data were suggestive of significant bacteriological contamination of divers following diving in polluted waters.

The study reported here was designed to assess the significance of recovery of potential waterborne pathogens from divers after diving by determining whether a specific immune response developed. We recruited experienced divers and inexperienced divers in training working in the Chesapeake Bay area for the study, and we describe the isolation of *Aeromonas* and *Pseudomonas* spp. from respiratory sites of the divers, their equipment, and the water itself. We prospectively monitored these divers over a 1-year diving period and report the prevalence and development of specific serum immunoglobulin G (IgG) responses.

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MATERIALS AND METHODS

Subjects. Divers from the Baltimore County Police Underwater Recovery Unit were recruited for this study. Subjects were experienced trained divers and divers who were in training. The experienced divers, 29 to 38 years of age, had cumulative diving experience in these waters of 53 years (mean, 7 years; range, 3 to 15 years). The naive divers, ages 26 to 34 years, had no previous diving experience in these waters but had fulfilled initial training requirements prior to diving at the police work sites. These new recruits had, on average, diving experience of 1 year at the time of this study (range, 0 to 2 years). No *Pseudomonas* or *Aeromonas* infections had been documented for any of these divers at the time of recruitment. Dives took place in the Middle River, Md., and the Delta, Pa., areas of the Chesapeake Bay. Written informed consent was obtained from all participants, as approved by the Institutional Review Board of the University of Maryland School of Medicine.

Specimen collection. Each dive took 30 min. Immediately before each dive and approximately 30 min after, the divers' noses, throats, ears, diving masks, and mouthpieces were swabbed and the specimens were placed in alkaline peptone water for transport. Samples from the top of the water column (1 m below the surface) and from river sediment were also collected for bacteriological analysis. Sampling methods for water and sediment analysis are described elsewhere (9).

For evaluation of acute immune responses (ARs), 20 ml of blood was collected from each diver, both before each dive and 30 to 60 days after. In addition, sera collected over a period of 18 months were used for studies evaluating the duration of specific immune responsiveness. All sera were stored at −20°C until use.

Bacteriological and immunological studies. *Aeromonas* and *Pseudomonas* spp. were isolated and identified by previously described methods (14). Immune responses to cell surface antigens of *Aeromonas* and *Pseudomonas* were determined by

TABLE 1. Recovery of *Pseudomonas* and *Aeromonas* spp. from water, sediment, diving gear, and respiratory tract sites of divers before and 30 min after a dive

Isolate recovery site	No. of samples positive/total no. of samples tested (%)[a]	
	Pseudomonas spp.	*Aeromonas* spp.
Water	6/7 (86)	5/7 (71)
Sediment	3/7 (43)	2/7 (29)
Diver (predive):		
Nose	1/49 (2)	0/49
Ears[b]	6/98 (6)	1/98 (1)
Throat	1/49 (2)	0/49
Gear[c]	6/98 (6)	1/98 (1)
Diver (postdive):		
Nose	16/49 (33)[d]	11/49 (22)[e]
Ears	47/98 (48)[f]	10/98 (10)[g]
Throat	6/49 (12)	1/49 (2)
Gear	13/98 (13)	6/98 (6)

[a] All significant P values for postdive recoveries compared with predive recoveries are given (chi-square).
[b] Right and left ear samples.
[c] Regulator and mask samples.
[d] $P = 0.002$.
[e] $P = 0.001$.
[f] $P < 10^{-8}$.
[g] $P = 0.01$.

a modification of a whole-cell extract assay (6). Each isolate was grown to a viable count of 10^{10} CFU/ml (by densitometer). Cells were pelleted at 12,000 × g for 10 min at 4°C and washed twice with phosphate-buffered saline (PBS), pH 7.4, containing 0.02% NaN_3. The cells were reconstituted to a final concentration of 10^9 CFU/ml in PBS-NaN_3.

An enzyme-linked immunosorbent assay was performed with this preparation at 100 μl per well, with alternating buffer-coated wells as controls. All sera were tested by endpoint titration with an initial dilution of 1:25, followed by twofold dilutions to 1:800. Negative control sera consisting of pooled sera from 10 6-month-old infants were used to determine absorbance cutoffs for a positive antibody response. A fourfold rise in IgG titer in postdive compared with predive samples was considered indicative of a seroresponse. A single positive reference serum for one isolate identified in the initial evaluation was used as the reference serum in subsequent assays to control for variability in test runs.

Statistical methods. All data comparisons were evaluated by chi-square analysis.

RESULTS

Subjects. Sixteen divers participated in these studies; nine were experienced divers and seven were naive divers. Experienced divers were evaluated in seven separate dives and naive divers were evaluated in six dives over the course of 1 year of study from November 1989 to November 1990. On average, each experienced diver participated in four dives (range, 1 to 7 dives) and each naive diver participated in three dives (range, 1 to 5 dives). In total, 49 separate diving experiences were studied.

Isolates recovered. The prevalence of *Aeromonas* and *Pseudomonas* organisms in the divers' noses, ears, throats, and gear before and after the study dives is presented in Table 1. Organisms were recovered from the respiratory tracts of divers prior to a dive in only 7 of 49 instances (14%). Two divers had organisms recovered from two respiratory sites prior to a dive. Evidence of persistent colonization was seen with only one experienced diver, who had *Pseudomonas aeruginosa* isolated from an ear canal at various times over a 4-month period. No naive diver ever had either *Pseudomonas* or *Aeromonas* organisms recovered prior to a dive. Gear was contaminated with these organisms prior to a dive in 7 of 98 (7%) cases. The predominant organism recovered prior to a dive was *P. aeruginosa*, which accounted for eight of nine (89%) respiratory tract isolates and six of seven (86%) gear isolates.

In contrast, 42 of 49 (94%) study dives resulted in isolation of either *Pseudomonas* or *Aeromonas* spp. from the divers' respiratory tracts at 30 min after the dive. The rate of recovery of these organisms from the noses and ears was significantly increased compared with predive levels, with the ears being the primary site of recovery. There was no difference in postdive recovery rates of naive divers and experienced divers. Although *Pseudomonas* and *Aeromonas* spp. were isolated from postdive throat and gear cultures, the rates of recovery from these sites were not significantly increased compared with predive recovery rates.

Postdive, *Pseudomonas* spp. accounted for 69 of 91 (76%) isolates recovered from the divers and 13 of 19 (68%) isolates recovered from diving gear. These results are in contrast to the equal rates of recovery of *Pseudomonas* and *Aeromonas* spp. from water and sediment. All *Pseudomonas* strains recovered were *P. aeruginosa*. *Aeromonas veronii* bv. sobria accounted for 26 of 36 (72%) *Aeromonas* isolates, with *Aeromonas caviae* making up 9 of 36 (25%) and *Aeromonas hydrophila* constituting only 1 of 36 (3%) *Aeromonas* isolates.

Immune responses. Acute and postdive sera were available for those divers participating in 33 of 49 (67%) individual dives. These paired sera, obtained within 2 months of a particular dive, were considered to be "acute immune response" sera because, for any one diver, only one dive would have occurred in that time interval. "Delayed immune responses" were those that occurred 6 months after any one particular dive.

ARs. ARs of experienced divers to 53 separate isolates (28 *P. aeruginosa* strains, 23 *A. veronii* bv. sobria strains, and 2 *A. hydrophila* strains) were measured over a 1-year period. ARs of inexperienced divers to 21 separate isolates (15 *P. aeruginosa* strains, 5 *A. veronii* bv. sobria strains, and 1 *A. hydrophila* strain) were measured over a 6-month period.

The ARs generated in experienced divers are shown in Table 2. For each diver, ARs to strains isolated from that same diver, other divers, water, and sediment were measured. Over the year of study, only 22 of 197 (11%) serologic responses of experienced divers showed evidence of a new immune response to organisms recovered immediately after a dive. There were equal rates of seroconversions in response to organisms recovered from divers' own bodies and organisms recovered from other team members, i.e., 12 and 14%, respectively. In addition, new responses were distributed equally between *Aeromonas* and *Pseudomonas* isolates. The seroresponse rates of these experienced divers did not significantly change over the course of a year, suggesting that these divers were constantly being exposed to new strains (data not shown; $P = 0.3$). The low rates of seroresponse to these recovered strains were probably due to the fact that 62 to 88% of the experienced divers already had significant immunologic reactivity to recovered isolates, which was evidenced by the presence of specific IgG titers of >100 to specific whole-cell antigens in acute serum samples. The one diver from whose ear *P. aeruginosa*

TABLE 2. Acute (within 2 months) and delayed (at 6 months) immune responses to whole-cell antigens of the *Pseudomonas* and *Aeromonas* isolates recovered from divers

Isolate recovery site	No. of positive immune responses[a]/total no. of samples tested (%)					
	Experienced divers		Inexperienced divers			
	Acute	IgG titer of ≥100[b]	Acute	IgG titer of ≥100[b]	Delayed	IgG titer of ≥100[b]
Water[c]	1/29 (3)	18/29 (62)	1/9 (11)	4/9 (44)	2/4 (50)	3/4 (75)
Diver[d]	3/22 (14)	17/22 (77)	1/19 (5)	0/19	4/6 (66)	4/6 (66)
Other divers[e]	18/146 (12)	129/146 (88)	8/56 (14)	23/56 (41)	8/34 (24)	20/34 (59)
Total	22/197 (11)	169/204 (83)[f,g]	10/84 (12)[h]	27/84 (32)[f,g]	14/44 (32)[h]	27/44 (61)[g]

[a] Number of serologic assays with a fourfold rise in titer. Data show combined responses to both *Pseudomonas* and *Aeromonas* spp.
[b] Presence in predive serum of a specific IgG titer of ≥1:100.
[c] Strains isolated from water and sediment.
[d] Isolates recovered from the diver's own body.
[e] Isolates recovered from other divers.
[f] $P < 10^{-8}$ (chi-square).
[g] $P = 0.003$ (chi-square).
[h] $P = 0.004$ (chi-square).

was recovered at various times over a 4-month period was in this group of divers.

In contrast, only 27 of 84 (32%) naive divers had evidence of prior immunologic recognition of water isolates recovered after a dive. This rate is significantly lower than that seen with experienced divers ($P < 10^{-8}$). In spite of this presumed increased immunologic susceptibility to potential water pathogens in the inexperienced divers, the rate of acute seroconversion in response to recovered isolates was similar to that observed for the experienced divers, i.e., approximately 14%.

Delayed immune responses. Using 12 strains isolated after the two initial dives made by four naive divers (in April 1990 and May 1990), we evaluated the development of specific immune responses to these isolates over 6 months. Whereas only a 4-of-44 (9%) rate of seroresponse to these isolates was achieved by the divers after the first 2 months of training, a 14-of-44 (31%) rate of response to these early-isolated strains was gained after 6 months of diving ($P = 0.0004$). In spite of this dramatic increase in specific IgG responsiveness at the end of 6 months of diving, naive divers still had a significantly lower rate of immune recognition of isolates present in water than experienced divers, i.e., 61% had IgG reciprocal titers of ≥100, compared with 83% of experienced divers ($P = 0.003$).

Duration of specific immunologic reactivity. In order to assess whether immunologic responses to water organisms persisted over time, we used three *Pseudomonas* and three *A. veronii* bv. sobria isolates recovered after the September 1989 dive and evaluated the specific IgG antibody levels in six divers. The responses are shown in Table 3. As can be seen, although there were a few (nine) cases in which specific antibody levels dropped during the 18-month survey, 46% (17 of 36) of the specific responses remained unchanged for at least 18 months. There were seven instances of a fourfold rise in antibody level, three of which were primary immune responses from divers who had no detectable antibody at the time of the initial isolation of the organism. Secondary immune responses were, in fact, observed with four divers, and these were all to only one isolate, an *A. veronii* bv. sobria strain isolated from a diver. This was the same strain that was responsible for a delayed primary immune response (detected in June 1991) in the diver from whom it was originally isolated in September 1989. These data suggest that certain organisms persist in waters and are able to elicit new immune responses, even in experienced divers.

TABLE 3. Duration of specific immunologic reactivity in six experienced divers

Isolate recovery site	No. of positive samples/total no. tested (%)		
	Predive specific IgG titer of ≥100 in September 1989	Rise (≥4-fold) in titer by June 1991	Drop (≥4-fold) in titer by June 1991
Sediment	5/6 (83)	1/6 (17)	3/6 (50)
Diver	2/2 (100)	1/2 (50)	0/2
Other divers	25/28 (89)	5/28 (18)	6/28 (21)

DISCUSSION

Although previous studies have revealed a high rate of mucosal or skin surface colonization by gram-negative enteric bacteria immediately after diving in polluted waters, the impact of such exposure has been difficult to assess (1, 2, 5, 10, 15). To determine whether this exposure had any measurable biologic effects, we investigated whether specific systemic IgG antibody to organisms recovered from divers, their equipment, or the diving site is present or develops over the course of a year of diving.

The rates of isolation of *Pseudomonas* and *Aeromonas* spp. from the respiratory tracts of divers before and after a dive were similar to those reported from our earlier studies as well as those reported by other investigators (1, 5). Although only very few divers had persistent oral and respiratory colonization with these organisms, we demonstrated that there was significant systemic immunologic recognition of them. The generation of specific seroresponses to waterborne pathogens is probably dependent, in large part, on the degree of exposure, that is, the number of dives a diver makes at a particular site. The majority of experienced divers had previous exposure to these organisms, which was evidenced by preexisting specific IgG antibody to 62 to 88% of the isolates recovered. Fewer divers in training than experienced divers demonstrated previous immunologic experience with the *Aeromonas* and *Pseudomonas* spp. recovered in this study, in which only 32% of the naive divers had levels of IgG specific antibody to these organisms that were >1:100, compared with 83% of the experienced divers. That diving specifically influenced the seroreactivity of new diving recruits is shown by the dramatic increase in specific seropositivity rates over the course of 6 months from 32 to 61%. Variables, other than number of dives made in a particular site over time, that could be responsible

for the generation of a specific immune response are seasonal variations in the water environment that can alter the concentrations of certain organisms and the length of time spent in the water during a dive. Such seasonal variations in organism concentration have been described for *Aeromonas* organisms (4). All dives in this study took place for 30 min, so we were unable to evaluate this variable. Whether the development of clinical signs of infection can be correlated to the development of an immune response will be the subject of future research.

Several other aspects of this study should be commented on. The first is that the recovery of a few selected organisms from water, sediment, or the respiratory tract of a given diver gives an incomplete picture of the true extent of that diver's exposure. Twelve to 24% of new seroresponses were to organisms recovered from other divers. Whether other mucosal sites such as the gastrointestinal tract would offer additional sources of infection could not be determined by the design of this study. In addition, because we did not measure mucosal immune responses to these recovered organisms, we may be underestimating the true extent of the microbiologic exposure of the divers.

The persistence of specific antibody in experienced divers, as evidenced by the maintenance of titers of specific antibody to organisms recovered after dives 18 months earlier, was noted. Whether continuous diving is a requisite for the persistence of specific antibody is not known.

The rates of seroconversion in response to recovered isolates were low, on the order of 11 to 12% per dive for both naive and experienced divers, suggesting a low level of infectivity of these organisms under diving conditions in which a diver wears a hood and wet suit. It is interesting to speculate what the rate would be for totally unprotected divers. Most importantly, this rate of seroconversion did not change over time, even for experienced divers, suggesting that there is a continuous turnover of biotypes or species in the water environment. These results suggest that, in cases in which systemic antibody may be important for protection against potential pathogens in an aquatic environment, there may be a period of susceptibility. For organisms whose distribution turns over rapidly, the period of susceptibility may be continuous. For naive divers, however, the range of susceptibility is greater. At present, however, the importance of systemic antibody in protection against these organisms in divers is not known. In addition, translation of this susceptibility to risk in both naive and experienced divers is very important and needs to be elucidated in future studies.

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