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Predictive value of anti-cell and anti-human immunodeficiency virus (HIV) humoral responses in HIV-1-exposed seronegative cohorts of European and Asian origin

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Unconventional immune responses have been demonstrated in individuals who, despite repeated exposure to human immunodeficiency virus (HIV) infection, remain seronegative. As environmental exposure to pathogens and genetic background may modulate immune responses differentially, one Italian and two Asian populations of HIV-1-exposed seronegative individuals were studied. In serum samples from each group, IgG to CCR5, IgG to CD4 and IgA to gp41 were measured, which were previously described as markers of unconventional immunity in HIV-exposed seronegative Caucasians. Given the importance of conformational epitopes in virus–cell interactions, IgG to CD4–gp120 complex was also measured. It was found that markers of HIV exposure were present in all populations studied. HIV-specific humoral responses (IgA to gp41 and IgG to CD4–gp120 complex) were extremely significant predictors of HIV exposure ($P < 0.0001$ in both cases), whereas the predictive values of anti-cell antibodies (anti-CCR5 and anti-CD4) varied between populations. Evidence is provided for the correlation of these differences with route of exposure to HIV and level of natural antibodies to cross-reactive microbial antigens. In conclusion, exposed seronegative individuals of ethnically different origins display similar signs of HIV-dependent unconventional immunity. A specific relevance must be attributed to different innate and acquired factors.

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INTRODUCTION

The host immune response can protect from both human immunodeficiency virus (HIV) acquisition and disease progression. The immune response to HIV and HIV-related antigens has been studied extensively, both in individuals who remain seronegative despite repeated viral exposure [exposed seronegative (ESN) subjects] and in subjects who maintain a benign evolution of HIV infection over several years [long-term non-progressors (LTNPs)]. Indeed, specific immunological peculiarities have been identified in ESN individuals, which are partly shared by LTNPs. Altogether, these findings suggest that viral exposure may occur in ESN subjects, but it does not progress to overt infection, nor does it cause a positive response in any standard assay for HIV detection. Indeed, extraordinarily low levels of HIV

infection have been recently described in ESN individuals (Zhu *et al.*, 2003).

Among HIV-protective factors, a genetic polymorphism of the CCR5 coreceptor gene has been reported (Liu *et al.*, 1996); however, this mutation cannot explain all cases of natural resistance to HIV infection (Rugeles *et al.*, 2002), thus suggesting that other factors may be involved. In fact, several antigen-specific responses to viral proteins described in ESN individuals could contribute to resistance to HIV infection. These include humoral immune responses, including IgA to Env (Beyrer *et al.*, 1999; Clerici *et al.*, 2002; Devito *et al.*, 2000a, b; Kaul *et al.*, 1999; Lo Caputo *et al.*, 2003; Mazzoli *et al.*, 1997; Shacklett *et al.*, 2002) and anti-cell antibodies, namely anti-human leukocyte antigen (HLA) (Beretta *et al.*, 1996; Lopalco *et al.*, 2000b), anti-CD4

(Burastero *et al.*, 1996; Lopalco *et al.*, 1999) and anti-CCR5 (Ditzel *et al.*, 1998; Lopalco *et al.*, 2000a). This aspect came strikingly to light in the experimental model of vaccination in non-human primates with cellular vaccines, where a relevant carry-over of membrane molecules was included in the immunogen, which was probably responsible for the induced protection (Lehner *et al.*, 1992). Moreover, cellular immune responses have also been described in ESN individuals (CD8 and CD4 T cells specific to HIV epitopes) (Biasin *et al.*, 2000; Furci *et al.*, 1997a, b; Kaul *et al.*, 2001; Makedonas *et al.*, 2002; Mazzoli *et al.*, 1997; Rowland-Jones & McMichael, 1995; Shacklett *et al.*, 2002; Shearer & Clerici, 1996). The study of this intriguing puzzle may pave the way to the design of anti-HIV strategies that are aimed at obtaining sterile immunity or improved viral control.

Here, we describe for the first time the characterization of several parameters of unconventional humoral immunity that were previously studied by our laboratories. These markers are specific to both cellular and HIV antigens, and were studied in three independent cohorts of ESN individuals with different exposure risks and geographical origins.

METHODS

Study populations. Serum samples from HIV-1-exposed seronegative (ESN) individuals belonging to three different populations, one Caucasian (Italian) and two Asian (Vietnamese and Cambodian), were analysed.

ESN individuals were defined as being seronegative by conventional ELISA (Wellcozyme HIV-1 recombinant), Western blot assays for HIV antibodies (HIV-1 blot 2.2; Diagnostic Biotechnology) and detection of HIV DNA (Amplicor; Roche) and RNA (Branch DNA; Bayer). Cut-off values for these standard assays are 50 copies of HIV DNA in 1 000 000 blood mononuclear cells and 50 copies of HIV RNA (ml plasma)⁻¹, respectively.

Sexually exposed. Italian heterosexual couples that were discordant for HIV serostatus were enrolled in the study. In each couple, one partner was infected with HIV whereas the other was not, despite a prolonged history of penetrative sexual intercourse without condom use (and no other risk factors). Inclusion criteria required a history of penetrative sexual intercourse without a condom, at least twice per week and for at least 2 years, for the majority of the subjects. Unexposed Italian controls (NESN) were recruited among healthy blood donors. Notably, all individuals included in the Italian cohort had been living in the same geographical area (Milan metropolitan area) for at least 10 years. Controls and ESN subjects were age- and sex-matched (Table 1).

Cambodian serodiscordant couples (SC) were recruited at the Anonymous and Free Voluntary Counseling and Testing Center of the Pasteur Institute of Cambodia (Nguyen *et al.*, 2003). For Cambodian ESN subjects, because of the difficulty of knowing the duration of exposure, an alternative criterion of inclusion was that the infected partner had <350 CD4 T cells mm⁻³. In a retrospective analysis, most infected partners showed a lower CD4 count of 1–200 cells mm⁻³ with more than 2 years of common life. Unexposed Cambodian controls were recruited among people coming for pre-marital tests. As for sexually exposed individuals, there were no differences between HIV-1-discordant and -concordant couples in age,

Table 1. Characteristics of the study populations

Population	n	Age range (years)	Sex	Risk factor
ESN				
Cambodian	51	19–51	42f, 9m	Heterosex
Vietnamese	37	39–58	4f, 33m	IVDU
Italian	61	30–48	35f, 26m	Heterosex
NESN				
Cambodian	50	16–42	42f, 8m	–
Vietnamese	32	26–60	2f, 30m	–
Italian	50	35–51	31f, 19m	–

common-life duration, sexual intercourse, condom usage or frequency of circumcision or STDs.

Systemically exposed. Vietnamese ESN subjects with a history of more than 10 years of high-risk intravenous drug use (IVDU) were included, presenting nearly 100% prevalence of other blood-transmitted viral infections, including hepatitis B virus, hepatitis C virus and human T-lymphotropic virus 1 (Truong *et al.*, 2003). Vietnamese healthy control subjects were recruited among Red Cross voluntary blood donors. Although the most suitable controls for Vietnamese ESN subjects would be Vietnamese individuals exposed systemically to non-HIV viruses, unfortunately this option could not be carried out. All of the study participants gave their informed consent and completed a questionnaire regarding the frequency and types of risk behaviours. Each was given counselling and information about HIV disease and safe drug or sex practices.

The study was approved by the Ethics Committee of the San Raffaele Scientific Institute, Milan, Italy; the National Ethics Committee of Cambodia; and the Ethics Committee of Binh Trieu Hospital, Ho Chi Minh City, Vietnam.

All blood samples were collected and frozen in the country of origin of each population. In order to reduce inter-test variability, samples were thawed and analysed simultaneously for each given parameter. All of the assays were done by a person blinded to the clinical status of the subjects.

All study participants were negative for the clinical and routine laboratory parameters of systemic and organ-specific autoimmunity, including antibodies to DNA, extractable nuclear antigens, mitochondria, the Fc fragment of IgG (rheumatoid factors) and thyroglobulin.

In order to exclude the possibility that the samples we analysed were taken during seroconversion, we only used samples from individuals who remained seronegative for at least 1 year after the date of bleeding.

Anti-CCR5 antibodies assay. The assay was performed as described previously (Lopalco *et al.*, 2000a). Briefly, a two-step gradient was created as follows: the lower layer consisted of fetal calf serum (FCS) containing 10% sucrose; the upper layer consisted of 80% silicone and 20% mineral oil (both from Sigma-Aldrich). Purified CD4 cells were incubated with appropriate dilutions of serum/plasma and/or IgG, purified and incubated with ¹²⁵I-labelled MIP1β. Unbound radioactivity was separated by centrifugation on the two-step gradient in 0.3 ml tubes (Nunc). Bound radioactivity in the cell pellets was measured by a gamma counter. A specificity control, consisting of a 100-fold excess of unlabelled MIP1β, was included in all experiments. Binding of the ¹²⁵I-labelled MIP1β to activated CD4 cells ranged between 1000 and 6000 c.p.m. An anti-CCR5 IgG assay was also performed by using 8 × 10⁴ CXCR4- or CCR5-transfected

U87 cell lines resuspended in 200 µl RPMI (Gibco Life Technologies), which were seeded and incubated for 2 days at 37 °C to obtain a cell monolayer. Serum samples taken from both ESN and NESN subjects were diluted 1/10 and incubated on the cell monolayer and ¹²⁵I-labelled sheep anti-human IgG antibodies (Amersham Biosciences) were added. Unbound radioactivity was separated by centrifugation on a two-step gradient as described above (Lopalco *et al.*, 2000a).

Anti-CD4 antibodies assay. The titre of IgG antibodies to human CD4 was measured by using a validated ELISA (Lopalco *et al.*, 1999). Briefly, recombinant CD4 [obtained from the National Institutes of Health (NIH) AIDS Reagent Program, Division of AIDS, NIAID, NIH, USA] was coated at 5 µg ml⁻¹ in PBS for 18 h at 4 °C on 96-well plates (Maxisorp; Corning). Sera to be tested were diluted 1/300 in 0.05% PBS/Tween 20 (Sigma). Each assay was run in quadruplicate. After 2 h incubation followed by washing, affinity-purified, alkaline phosphatase-conjugated goat antibody to human IgG (ICN Biomedicals) was added and the reaction was subsequently developed with *para*-nitrophenyl phosphate (Sigma). The amount of anti-CD4 IgG binding activity was expressed as OD₄₀₅. When anti-CD4-gp120 complex antibodies were to be measured, CD4 was pre-incubated with HIV BaL strain gp120 (NIH AIDS Reagent Program, Manassas, VA, USA) in equimolar amounts [2.6 µg gp120 (µg CD4)⁻¹] for 30 min on ice. All subsequent steps remained unchanged.

Measurement of inhibition of CD4 binding by homologous and heterologous antigens by cytofluorimetric analysis. In order to study the specificity for CD4 of IgG antibodies from different groups of ESN and NESN subjects, pools were prepared from people included in this study, using 25 µl of each serum. IgG fractions were isolated from pooled sera by affinity chromatography with Protein G-Sepharose (Amersham Biosciences) according to the manufacturer's instructions and brought to a final concentration of 1 mg ml⁻¹ in PBS. ESN sera were chosen from individuals positive for anti-CD4 antibodies (nine subjects) (Lopalco *et al.*, 1999), whereas NESN sera were chosen from individuals with IgG to CD4 values above the 75th percentile (12 subjects). The IgG to CD4 binding activity of each fraction was evaluated by cytofluorimetric analysis with a murine cell line transfected permanently with human CD4 (3T3.T4) (NIH AIDS Reagent Program). Purified IgG was pre-adsorbed onto wild-type NIH 3T3 cells (ATCC, Manassas, VA, USA) (5 × 10⁶ cells, in rotation at 4 °C for 1 h) to lower the background binding on 3T3.T4 cells. Each fraction was then incubated with soluble CD4 (5 µg ml⁻¹), Fc fragment of human IgG (10 µg ml⁻¹), human heat-shock protein 60 (HSP60) (10 µg ml⁻¹) or BSA (5 µg ml⁻¹) as a control. Subsequently, each soluble antigen-adsorbed aliquot was added to the 3T3.T4 cell line (5 × 10⁵ cells in a 100 µl reaction volume, 30 min on ice). Binding of IgG to 3T3.T4 cells was detected with a polyclonal, fluorescein isothiocyanate-conjugated goat antibody to human IgG (ICN Biomedicals). Analysis was performed with a FACScalibur cytofluorimeter (BD Biosciences). The control samples were 3T3.T4 cells reacted with the fluoresceinated secondary antibody only. Readings with controls were set to span fluorescence channels 1–10. To make the system more sensitive, for each non-adsorbed IgG fraction (positive control), a dilution was preliminarily identified if it yielded a binding activity spanning fluorescence channels 20–40. Inhibition of binding was expressed as percentage mean fluorescence intensity (MFI) in the given sample compared to MFI in the positive control.

Enzyme immunoassay detection of gp41-specific antibodies. The presence of gp41-specific IgA was evaluated by ELISA as described previously (Clerici *et al.*, 2002). In brief, ELISA was performed by using gp41 recombinant protein for coating. IgA binding was revealed with horseradish peroxidase-conjugated rabbit polyclonal antibody to human IgA (Dako).

Statistical analysis. Preliminary tests for normality (Shapiro–Wilk, Kolmogorov–Smirnov) consistently showed a skewed distribution of values for all parameters we measured. Thus, analysis of results was performed with non-parametric tests. Cumulative results were expressed as medians and quartiles. Subjects within the ESN groups were considered to be positive for each given variable when values fell above the 95th percentile of the distribution of values in the respective NESN group within each population. The χ^2 test was used to compare ESN and NESN individuals for each variable and within each population, and values of *P* below 0.05 were considered to be significant. The odds ratio for case-control analysis was used to calculate the risk of belonging to the ESN group when positivity for the given parameter was present; 95% confidence limits are shown. The Mantel–Haenszel test was used to estimate the common odds ratio for each parameter, when considering cumulatively the values obtained from the three populations that were studied. The Breslow–Day test was used to assess the homogeneity of the odds ratios in the different populations and gave non-significant results in all cases. Finally, NESN individuals of different ethnic origins were analysed in parallel (inter-cohort comparison) by comparing a single parameter from one cohort against the corresponding parameter of each other cohort. For this purpose, the Kruskal–Wallis test was used and post hoc comparisons were adjusted with the Bonferroni procedure. Calculations were performed by using the SAS System software.

RESULTS

Demographic characteristics of the studied populations

A different genetic and/or environmental background can influence the immune response and, consequently, the susceptibility to HIV infection in developing countries versus Europe or North America (Bentwich *et al.*, 1995; Lawn *et al.*, 2001; Levy *et al.*, 2003). On this basis, Caucasian (Italian) and Asian (Cambodian and Vietnamese) subjects were included in the present study. Moreover, in order to compare sexually and systemically HIV-1-exposed individuals for immune correlates of HIV-1 resistance, subjects with different HIV risk factors were included. Namely, Italian and Cambodian ESN subjects were sexual partners of HIV-seropositive patients, whereas the risk factor for Vietnamese ESN subjects was IVDU. Demographic and clinical characteristics of the studied subjects are shown in Table 1. Italian ESN subjects were characterized for the presence of CCR5-32 alleles. Non-homozygous subjects were identified within the studied individuals (data not shown).

Predictive power of HIV exposure by humoral immune-response analysis in different groups of ESN subjects

The presence of IgG to CCR5, IgG to CD4 and IgA to gp41 was previously correlated with protection in Caucasian ESN individuals (Burastero *et al.*, 1996; Clerici *et al.*, 2002; Lopalco *et al.*, 2000a). Here, we simultaneously evaluated these three parameters in parallel in Asian and Caucasian populations. The relevance of the initial steps in virus–cell interaction has recently been highlighted by the possibility of interfering with several viral processes through protective

Table 2. Intergroup comparison among NESN subjects

The Kruskal–Wallis test yielded $P \leq 0.0001$ for all comparisons. Post hoc multiple comparisons were performed by using Dunn's test. NS, Not significant.

Antibodies	Median values			Kruskal–Wallis test		
	Cambodia	Vietnam	Italy	Vietnam vs Cambodia	Cambodia vs Italy	Vietnam vs Italy
IgG to CCR5*	0.43	0.05	0.68	NS	<0.05	<0.05
IgG to CD4†	0.81	1.03	0.23	NS	<0.01	<0.01
IgA to gp41†	0.266	0.203	0.140	<0.05	<0.01	<0.01
IgG to the CD4–gp120 complex†	0.78	1.02	0.24	NS	<0.01	<0.01

*Percentage binding.

†OD₄₀₅.

immune responses against strictly conformation-dependent epitopes that are exposed during viral docking and entry. Thus, we also evaluated antibodies to gp120-dependent CD4 epitopes as further potential immune correlates of HIV exposure in all studied populations. Cell-associated self-antigens that are involved in HIV entry (CD4, CCR5) and strictly virus-specific epitopes (gp41, gp120–CD4 complex) were evaluated separately. Firstly, in order to compare the distribution of levels of each single parameter in the NESN control populations, the Kruskal–Wallis test was used to compare single parameters against the corresponding parameter of each other group and post hoc comparisons were adjusted with the Bonferroni procedure. Non-significant differences were found between the two Asian cohorts for all considered parameters. In contrast, highly significant differences were found when comparing the NESN subjects from Italy with their counterparts from both Vietnam and Cambodia, with the exception of anti-CCR5 antibodies, which were relatively homogeneous in the three populations. In particular, extremely significant differences were found when considering IgG to CD4 and to gp120–CD4 complex, as well as IgA to gp41 (Table 2). These results prompted us to evaluate the levels of natural antibodies in the different populations, in order to explore the possibility that polyreactive antibodies could affect the single tests that we used differently (see below). Secondly, in order to verify whether these humoral parameters can be considered as specific predictive markers of HIV exposure, we calculated the odds ratio, which measures the magnitude of divergence between HIV-exposed versus non-exposed subjects, for each variable.

In Cambodians, the odds ratios of anti-virus antibodies (both IgA to gp41 and IgG to CD4–gp120 complex) were extremely statistically significant ($P < 0.0001$). The odds ratios of IgG to CD4 were significant ($P = 0.0072$), whereas those of IgG to CCR5 were not (Table 3).

Table 3. Comparisons of antibodies in ESN and NESN individuals within each group

Odds ratios per group and common odds ratio. For (a–c), P indicates probability according to Cochran–Mantel–Haenszel statistics. Odds ratios were calculated by using the Mantel–Haenszel test for case-control analysis. NS, Not significant. For (d), the Breslow–Day test was used to assess the homogeneity of the odds ratios and yielded non-significant results in all cases. Common odds ratios were therefore calculated. P indicates probability according to Cochran–Mantel–Haenszel statistics. Odds ratios were calculated by using the Mantel–Haenszel test for case-control analysis.

	P	Odds ratio (confidence limits)
(a) Antibodies (Cambodia)		
IgG to CCR5	NS	2.66 (0.49–14.41)
IgG to CD4	0.0072	12.15 (1.38–106.48)
IgA to gp41	NS	18.58 (4.00–84.86)
IgG to the CD4–gp120 complex	<0.0001	5.31 (1.08–26.13)
(b) Antibodies (Vietnam)		
IgG to CCR5	0.0455	7.00 (0.81–60.00)
IgG to CD4	NS	3.68 (0.39–34.56)
IgA to gp41	0.0453	7.00 (0.81–60.00)
IgG to the CD4–gp120 complex	0.0259	5.31 (1.08–26.13)
(c) Antibodies (Italy)		
IgG to CCR5	0.0021	8.54 (0.98–156.41)
IgG to CD4	0.0078	6.78 (1.41–32.36)
IgA to gp41	<0.0001	53.95 (6.92–420.42)
IgG to the CD4–gp120 complex	0.0143	6.00 (1.24–29.98)
(d) Parameters		
IgG to CCR5	0.0033	5.75 (1.60–20.58)
IgG to CD4	0.0001	6.89 (2.30–20.64)
IgA to gp41	<0.0001	21.41 (7.56–60.62)
IgG to the CD4–gp120 complex	<0.0001	8.50 (3.22–22.38)

In Vietnamese IVDU subjects, the odds ratio of IgA to gp41 antibodies was barely significant ($P=0.0453$) and that of anti-CD4–gp120 complex antibodies was significant ($P=0.0259$). As for anti-cell antibodies, the odds ratio of IgG to CCR5 was barely significant ($P=0.0455$), whereas that of IgG to CD4 was not (Table 3).

In Italian ESN subjects, the odds ratio of IgA to gp41 antibodies was extremely significant ($P<0.0001$) and that of anti-CD4–gp120 complex antibodies was significant ($P=0.0143$). As for anti-cell antibodies, both the odds ratios of IgG to CCR5 and of IgG to CD4 were significant ($P=0.0021$ and 0.0078 , respectively) (Table 3).

In order to assess the homogeneity of the odds ratios in the different populations, the Breslow–Day test was preliminarily performed, which indicated that the common odds ratio could be extrapolated from the cumulative analysis of each parameter in all studied populations. Strikingly, the significance of the predictive power of each parameter was higher than those observed in each single population (Table 3). In particular, anti-virus antibodies were extremely significant predictors of HIV exposure ($P<0.0001$).

In order to express the data in a graphical way, we used box-and-whisker plots. In this representation, the box indicates the lower and upper quartiles and the central line represents the median. The points at the ends of the ‘whiskers’ indicate extreme values. Single dots in ESN graphs indicate values above the extreme value of the respective NESN group (Fig. 1). Moreover, we arbitrarily extrapolated the proportion of ESN individuals positive for the presence of antibodies to each marker as the ratio between the number of individuals with values above the extreme NESN value in the corresponding population and the number of ESN subjects that were analysed.

The pattern of the distribution of IgG to CCR5 antibodies was similar in the three ESN populations. In particular, the proportion of ESN subjects with values above the extreme values of the corresponding NESN populations fell in a rather strict range (7.8–14.6%) (Fig. 1a).

Different levels of IgG to CD4 antibodies were displayed in NESN populations of Asian origin compared to Italians, as demonstrated previously by the Kruskal–Wallis test (Table 2, Fig. 1b).

This high background probably affected the comparison with the corresponding ESN individuals, although the proportion of ESN subjects with values higher than the NESN extreme values was similar (range, 2.4–7.8%) (Fig. 1b). IgG to CD4–gp120 complex, similarly to anti-CD4 antibodies, was higher in the NESN populations of Asian origin (Table 2, Fig. 1c). However, for this parameter, higher proportions of ESN subjects with values above the respective NESN extreme values were found (range, 13.1–17.6%). Notably, the mean value of the

proportion of ESN subjects with antibodies to CD4–gp120 complex was about three times higher (15.9%) than that of ESN subjects with antibodies to CD4 (5.5%).

For IgA to gp41 in ESN subjects, the proportion of positive subjects ranged from 17 to 57.3% (Fig. 1d). Notably, Vietnamese IVDU subjects, who were exposed systemically to HIV, showed lower level of IgA antibodies than the two populations of sexually exposed individuals. This result is evident in the odds-ratio test, which was barely significant in Vietnamese ESN subjects ($P=0.0453$), compared to the two sexually exposed groups ($P<0.0001$ in both Italian and Cambodian ESN subjects) (Table 3).

Studies on the specificity of the anti-CD4 response by purified IgG

In order to evaluate the possibility that different levels of cross-reactive antibodies in different populations could correlate with different levels of binding to the antigens that we included in our tests, dedicated assays were designed. Firstly, the IgG fraction of antibody was isolated from sera, in order to exclude interference by the highly cross-reactive IgM isotype. Secondly, competition assays were performed with antigens that were representative of self/non-self cross-reactive molecules (Burastero *et al.*, 1988; Casali *et al.*, 1987). Results of three separate experiments of inhibition with soluble antigens are shown in Fig. 2. Although the analysis is merely qualitative, it appears that IgG of individuals from both Cambodia and Vietnam were efficiently displaced from binding to CD4, not only by the homologous soluble molecule, but also by the heterologous antigens that we tested. This applied to both ESN and NESN subjects of these groups. In contrast, IgG of ESN and NESN individuals from Italy was displaced from binding by pre-incubation with soluble CD4, but not with soluble Fc of HSP60. Similar results were also observed with thyroglobulin, another autoantigen that is recognized by natural polyreactive antibodies (data not shown) (Nakamura *et al.*, 1988). A representative result is shown in Fig. 3 (control line). The CD4 binding of IgG purified from ESN individuals from Italy (Fig. 3, left panel) was reduced from an MFI of 32.6 to an MFI of 11.8 (–63.8%) by pre-incubation with soluble CD4, but remained unchanged following pre-incubation with the heterologous antigen HSP60 (MFI, 31.0) (–5%). In contrast, the CD4 binding of IgG from individuals from Cambodia (Fig. 3, right panel; MFI, 84.1) was similarly reduced by incubation with both soluble CD4 (MFI, 32.8) (–61%) and HSP60 (MFI, 33.8) (–59.8%).

DISCUSSION

Several immune responses to HIV antigens and to cell antigens have been reported in individuals who are exposed to HIV, but remain seronegative (ESN). These subjects are negative in all standard assays for detection of both antibodies to HIV and of viral RNA/DNA, yet they demonstrate with these traits that their immune system did come into contact with HIV.

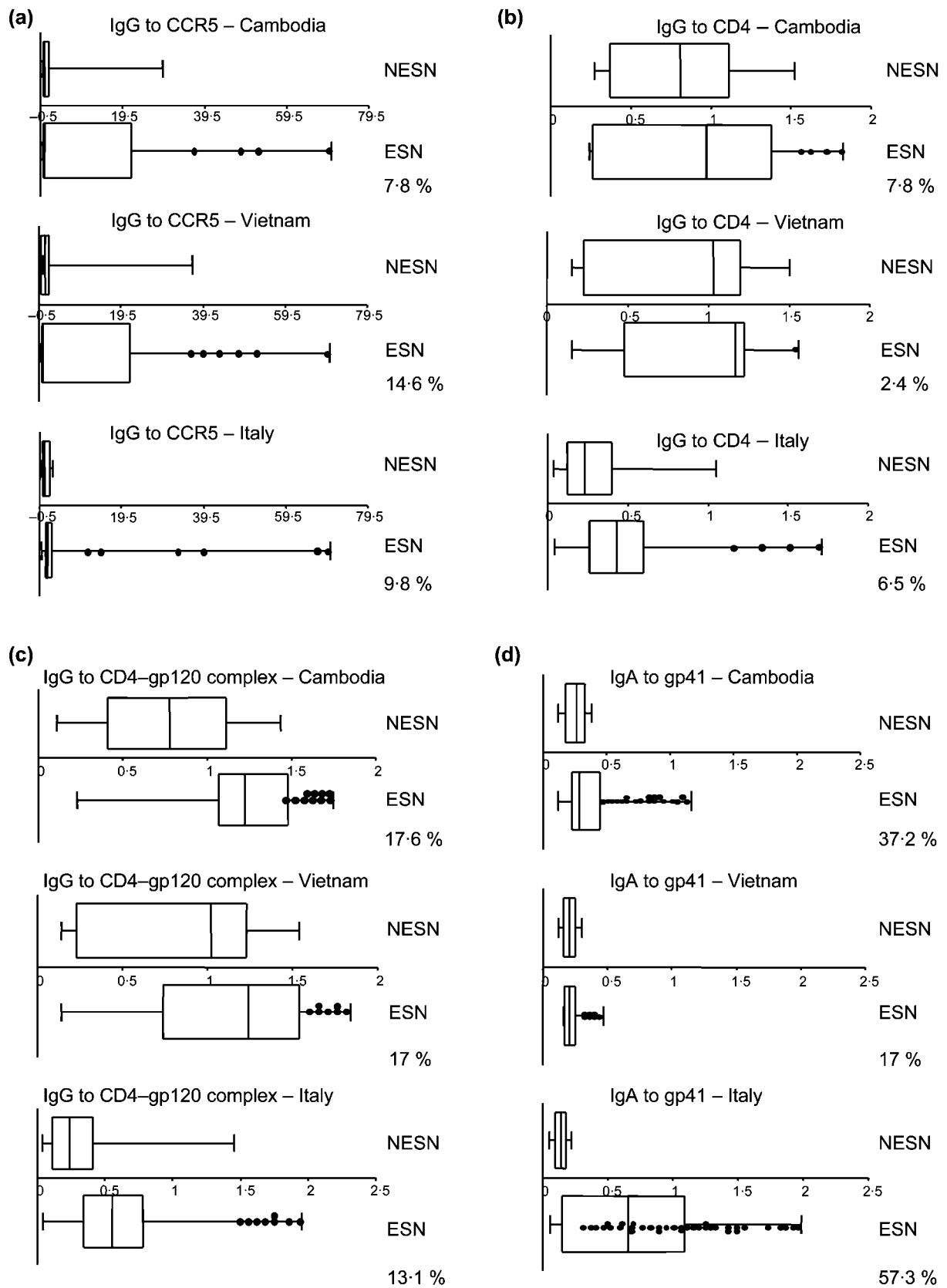


Fig. 1. Box-and-whisker representations of the values of the four considered markers of HIV exposure. The box indicates the lower and upper quartiles and the central line represents the median. Points at the ends of the 'whiskers' indicate upper extreme values. Single dots in the ESN graphs indicate values above the extreme value of the respective NESN group. The percentage of ESN individuals positive for the presence of antibodies to each marker has been extrapolated arbitrarily as the ratio between the number of individuals with values above the extreme NESN value in the corresponding population and the number of ESN subjects analysed. Values indicate the percentage competition for MIP1 β binding on CD4 lymphocytes (a), IgG binding activity expressed as OD₄₀₅ (b, c) and IgA binding activity expressed as OD₄₀₅ (d).

Different genetic backgrounds and different antigenic experience due to distinct environmental conditions can significantly modulate immune responses to microbes (Bentwich *et al.*, 1995). Thus, we decided to analyse humoral markers of HIV exposure that were previously identified in Caucasian ESN subjects in a large cohort of Italians and, in parallel, in two cohorts of Asian origin (Cambodian and Vietnamese subjects). Moreover, individuals with different routes of exposure to HIV were included, as Italians and Cambodians were sexually exposed, whereas Vietnamese were exposed by IVDU.

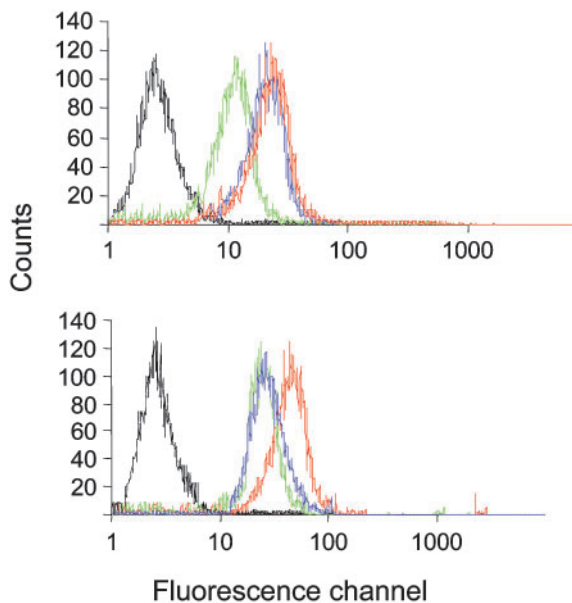


Fig. 2. Representative experiment of inhibition of IgG binding to CD4 by soluble CD4 and HSP60. Protein G-purified IgG fractions from pooled sera of ESN and NESN individuals from Italy (top) and Cambodia (bottom) were evaluated for IgG binding activity to CD4 by using FACS analysis and the NIH 3T3 human CD4-transfected cell line. Arbitrary channels of fluorescence intensity and cell counts are on the *x* and *y* axes. The red line corresponds to baseline CD4 binding activity and the black line to background fluorescence, i.e. the fluorescence of cells reacted with the anti-human IgG fluoresceinated probe only. The CD4 (green) and HSP60 (blue) lines correspond to the binding activity observed when the same IgG fraction was pre-incubated with either recombinant human CD4 (to evaluate homologous inhibition) or recombinant human HSP60 (to evaluate heterologous inhibition).

We made inter-group comparisons between NESN individuals in order to evaluate whether populations living in geographical areas with distinct lifestyles and social-health policies displayed different levels of the humoral immune parameters that we analysed, independently of specific exposure to HIV. This might be correlated, among other factors, with different levels of natural polyreactive

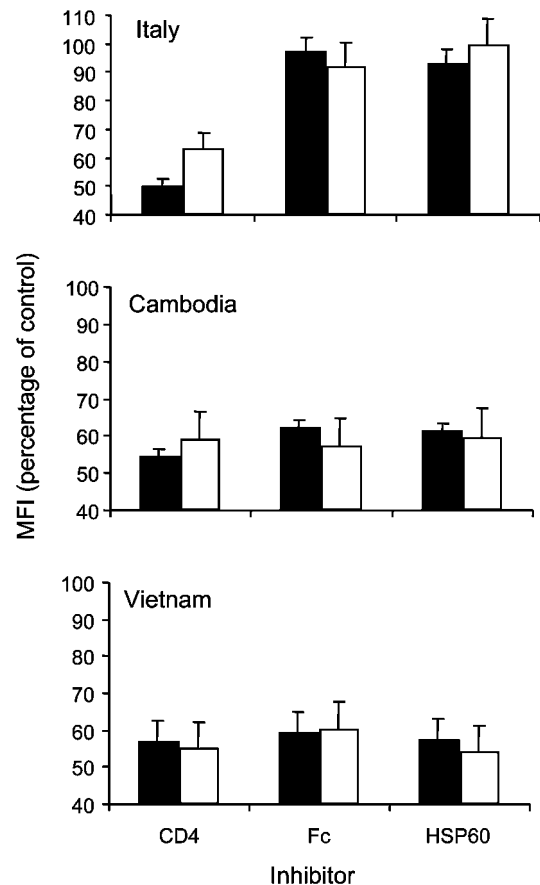


Fig. 3. Cumulative results of homologous- and heterologous-inhibition experiments. CD4-binding activity was evaluated as described in the legend to Fig. 2. The results are expressed as percentage mean fluorescence intensity (MFI, on the *y* axis) in comparison with the control (i.e. baseline CD4-binding activity). The IgG fractions were tested on human CD4-expressing cell lines after pre-incubation with recombinant human CD4, Fc fragment of human IgG and human HSP60 (as indicated on the *x* axis). The IgG fractions came from pooled sera of individuals (filled bars, ESN; empty bars, NESN).

antibodies. Indeed, all considered parameters were higher in both Asian populations than in the Italian group ($P < 0.0001$), with the exception of autoantibodies to CCR5, which were homogeneously distributed (Table 1) and tended to be higher in Italians than in Asians, although they did not reach significance. It is tempting to speculate that the well-known reverse correlation between autoimmunity (and allergy) and overall frequency of infections, which has been observed in both humans and animal models (Yazdanbakhsh *et al.*, 2002), might explain this result. Notably, IgG to CCR5 was detected with an assay that was specific for a single epitope, whereas all other assays used whole molecules and were therefore more susceptible to interference by natural polyreactive antibodies. Indeed, we confirmed this hypothesis by analysing a selected panel of sera. We found that an autoantigen, such as HSP60 (an example of an antigen recognized by natural polyreactive antibodies) (Pashov *et al.*, 2002; Potter *et al.*, 2000), could displace IgG from Cambodian and Vietnamese individuals from binding to membrane-bound CD4 as efficiently as soluble CD4 itself. Also, the Fc fragment of human IgG, which is recognized by 'natural' serological rheumatoid factor activity (Burastero *et al.*, 1988), could efficiently compete with CD4 binding; similar results were observed with thyroglobulin, as described previously (Nakamura *et al.*, 1988). In contrast, binding activity to membrane-bound CD4 by IgG to CD4 purified from Italians was specifically outcompeted by soluble CD4, but not by the heterologous antigens (Fig. 2).

Higher and frequent exposure to numerous infectious agents in poor countries, such as Cambodia and Vietnam, is probably inducing a general immune activation, as has been described in Africa (Rizzardini *et al.*, 1996, 1998). An increase of polyreactive antibodies consequent to immune activation may thus underlie the differences observed between Asian and Italian populations. Further studies are needed to test the level of immune activation in the Asian groups (e.g. by measuring parameters of T-lymphocyte and complement activation, cytokine production, etc.).

In order to verify whether the humoral parameters that we studied can be considered as specific predictive markers of HIV exposure, we calculated the odds ratio, which measures the magnitude of divergence between HIV-exposed versus non-exposed subjects for each variable.

On one side, a cumulative analysis of each parameter was performed in all studied populations (Breslow–Day test followed by Cochran–Mantel–Haenszel statistics), which allowed a strengthening of the power of predictivity of the markers (Table 3). On the other, qualitative analysis of each marker by box-and-whisker plots further allowed us to visualize the pattern of distribution of values within each ESN versus NESN group (Fig. 1). Taken together, both approaches demonstrate unequivocally that ESN subjects from cohorts recruited in distinct geographical areas, implying striking environmental and genetic differences,

do display immunological signs of HIV exposure, compared to their NESN counterparts.

Notably, anti-virus antibodies (IgA to gp41 and IgG to gp120-dependent CD4 epitopes) were more powerful predictors of HIV exposure than anti-cell antibodies (IgG to CD4 and CCR5) (Table 3). This observation suggests that this humoral immune response is strictly dependent on actual exposure to HIV. This may be due to: (i) an extremely low level of ongoing virus replication, as recently reported in a few ESN individuals (Zhu *et al.*, 2003); (ii) a previous event where single rounds of HIV replication took place, followed by complete viral clearance; or (iii) previous exposure to viral debris, in amounts and with modalities compatible with the elicitation of HIV-specific antibodies. On the other hand, non-HIV-specific events could also contribute to the induction and modulation of anti-cell humoral immune responses towards membrane molecules that are involved in HIV docking and entry. Indeed, our results also show that anti-cell antibodies must be considered as predictive markers of HIV exposure (Ditzel *et al.*, 1998; Lehner *et al.*, 1992, 1999; Lopalco *et al.*, 2000a).

We show here for the first time that the fine specificity of anti-CD4 antibodies in ESN individuals includes epitopes whose expression on CD4 molecules is triggered by binding to gp120. This is consistent with the exclusive mechanism for generation of the anti-CD4 response that was described previously in ESN subjects (Furci *et al.*, 1997a), which was strikingly different from that observed in HIV-positive individuals with anti-CD4 antibodies (Salemi *et al.*, 1995).

Moreover, although one study failed to detect the presence of HIV-specific mucosal IgA (Dorrell *et al.*, 2000), several other reports confirmed this observation in populations with different genetic backgrounds, including Kenyans, Thai commercial sex workers and an Italian SC cohort (Beyrer *et al.*, 1999; Clerici *et al.*, 2002; Devito *et al.*, 2000a, b; Kaul *et al.*, 1999; Lo Caputo *et al.*, 2003; Mazzoli *et al.*, 1997; Shacklett *et al.*, 2002). Taken together, these data confirm the heterogeneity of mechanisms leading to ESN status and stress the relevance of the route of exposure.

In conclusion, both anti-cell and anti-HIV antibodies are constant hallmarks in individuals who are exposed continuously to HIV infection, but remain persistently seronegative when evaluated with standard assays. We believe that we are providing important confirmation of the presence of these potentially protective antibodies in ESN individuals. Moreover, the present work is the first evidence that race and route of virus exposure may differentially affect the antibody specificity that is associated with ESN status. Finally, we show evidence of the nature of polyreactive antibodies and their role in generating and measuring the antibodies evaluated here. Overall, this work increases our understanding of innate and acquired immune responses that might protect against HIV-1.

The study of unconventional immunity in ESN subjects

needs to be further fostered, in order to identify potential strategies to sterile immunity or HIV-infection control. Our work also indicates that special caution is to be utilized to overcome the problems that are encountered when non-standard assays for detecting viral or cellular targets of cellular and humoral immune responses are applied to people with different ethnic origins and/or antigenic experience.

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