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SOLUBLE CD30 AND LYMPHOCYTE ACTIVATION GENE-3 (CD223), AS POTENTIAL SEROLOGICAL MARKERS OF T HELPER-TYPE CYTOKINE RESPONSE INDUCED BY ACELLULAR PERTUSSIS VACCINE

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T cell responses are involved in vaccine-induced immunity to pertussis but no easy-to-monitor, serological markers are available to assess these responses. The lymphocyte activation gene-3 (CD223) molecule is present on, and released by, activated T helper (Th) 1 cells, whereas CD30 molecules have been associated with Th2 immune responses. Starting from the recent knowledge of the cytokine profile induced by pertussis vaccination, we examined the levels of soluble (s)CD223 and sCD30 proteins in child recipients of acellular pertussis (aP) and diphtheria-tetanus (DT) vaccines and in children receiving DT vaccine only, as control. The correlation of the two proteins with specific antibody and T cell responses was assessed. The main findings are: i) sCD223 and sCD30 levels are inversely related, suggesting that the two markers are the expression of different and counter-regulated T-cell responses; ii) sCD30 level correlated with induction of T cell proliferation to pertussis vaccine antigens and antibody response to pertussis toxin. Overall, sCD30 and sCD223 levels seem to be promising candidate markers to assess the induction of Th-type responses in vaccine recipients.

The mechanisms underlying induction of immune protection from *Bordetella pertussis* infection are not completely understood (1). Data from experimental infections and, in part, clinical studies suggest that natural immunity as well as T and B memory cell compartments are involved, but the differential contribution of each of these components in the protection induced by vaccination or disease-free natural exposure to *B. pertussis* or the disease itself remains largely undetermined (1-10). A correlation between high titers of antibodies against some *B. pertussis* antigens and protection of household contacts of pertussis cases has been found (11-13), nevertheless, no direct correlation between serum antibody levels and protection from pertussis was found in several clinical trials with extended, carefully investigated, follow-up periods (14-18).

Since the discovery of T helper (Th)1 and Th2 lymphocyte subsets and their role in the regulation of immune response, studies have been intensely focused on the possibility of discriminating the prevalent expansion of CD4 lymphocytes with Th1 or Th2

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profile, in consideration to the differential role of each subset for vaccine efficacy. Th1 or Th2 are mainly discriminated through the pattern of their secreted cytokines, in that Th1 subsets secrete interferon gamma (IFN-γ) but not IL-4, while Th2 subsets secrete IL-4, IL-5, IL-6, IL-10 and IL-13 but not IFN-γ However, a mixed pattern of cytokines is often secreted by a CD4 subset defined as Th0 (19-20).

In the specific case of pertussis vaccines, the complexity of the mechanisms underlying immune protection following vaccination is demonstrated by the observation that equally protective primary vaccinations such as those achieved by whole cell (wP) or acellular (aP) vaccines induce different Th cytokine profiles (3, 6-7, 9-10). Particularly, our previous data from the double blind, randomized, controlled clinical trial of pertussis vaccine efficacy in Italy, where children received an aP or a wP vaccine, highlighted a higher Th2 response in aP-vaccine recipients (3). In contrast, a fairly polarized Th1 response, somewhat mimicking the immunity induced by natural infection, was measured in wP vaccines (3-4, 6, 10, 21-23). In this context, the availability of serological markers of T cell responses would be of great help.

Several surface molecules have been reported to be differentially expressed by Th1 and Th2 cells. In particular, the lymphocyte activation gene-3 (CD223) molecule has been shown to be expressed on, and released by, activated IFN-γ producing Th1 cells (24), whereas the CD30 molecule has been shown to be expressed on, and released by, activated IL-4 producing $CD4^+$ Th2 cells (25-26). The determination of these soluble factors could provide insight into immune responses to vaccines in general, and particularly help clarify differences in the mechanisms of pertussis vaccines, which can induce both Th1 and mixed Th1/Th2 responses (3, 6-7, 9-10). Despite this, to our knowledge, neither factor has ever been determined in the context of infant vaccination.

Thus, the aims of this study were, first, to determine whether soluble (s) CD223 and sCD30 were present and measurable in early childhood; second, to examine whether sCD223 and sCD30 levels were related to adaptive immune responses against *B. pertussis* antigens in vaccine recipients; third, to examine modulations of these soluble factors in relation to vaccination against pertussis.

MATERIALS AND METHODS

Subjects

Plasma and sera were collected from children participating in the double blind, randomized controlled clinical trial of pertussis vaccine efficacy performed in Italy. The general study design and details of this benchmark clinical trial has been reported elsewhere (2, 14, 18). For the present study, two groups of children were randomly selected for assessment of sCD30 and sCD223 markers from all those participating in a clinical trial of pertussis vaccine efficacy (2, 14), i.e. 76 children who received the aP-DT vaccine and 30 who received the DT vaccine only. Informed, written consent was obtained by parents or guardians of the children enrolled in this study. The original study was approved by the bioethical committee of the Italian Trial of Pertussis Vaccine Efficacy.

The pertussis vaccine contained chemically inactivated pertussis toxin (PT), filamentous haemagglutinin (FHA) and pertactin (PRN) at 25, 25 and 8 μg, respectively, while the DT vaccine component contained 25 Lf of diphteria toxoid and 10 Lf of tetanus toxoid. Sera or plasma were collected before vaccination, at 2 months of age, and one month after the completion of the primary vaccination schedule, when the children were 7 months old.

T cell mediated immune (CMI) and antibody response definitions

CMI response, measured as antigen specific T cell proliferation (2), was assayed in 43 children, 33 aP-DT- and 10 DT- vaccine recipients (2). A CMI response was considered positive when the difference between the antigen-stimulated PBMC culture and the unstimulated one was at least $3x10^3$cpm, and the mean stimulation index was at least 4.

The serological response was measured in 106 children, 76 aP-DT- and 30 DT- vaccine recipients (2, 18). A serologic response to each pertussis antigen was defined as positive when the Enzymatic Immune Assay (EIA) value was 4 times higher than the minimal level of detection. The minimal level of detection was set at 2 U/ml for IgG to PT and FHA and 3 U/ml for IgG to PRN (2, 18).

Determination of sCD223 and sCD30

Serum sCD223 level was assessed by an immunoenzymatic assay, with little modification, from a previously reported protocol (27). Briefly, purified anti-CD223 11E3 (IgG1) monoclonal antibody (mAb) (IMMUTEP, Orsay, France) was used as capture antibody and anti-CD223 17B4 (IgG1) biotinylated mAb as revealing antibody (courtesy of Michel Dreano, Serono International SA, Switzerland). Purified CD223 protein was used as reference standard (IMMUTEP, Orsay, France) and data are expressed as ng/ml. The ELISA sensitivity was 0.07

ng/ml corresponding to 0.163 ± 0.004 O.D. (mean ± SE of 9 determinations). sCD30, was measured by commercial ELISA from Dako (Glostrup, Denmark). Data are expressed as U/ml, the lower sensitivity limit (1 U/ml) corresponding to the mean absorbance plus 2 SD of 20 measurement of the 0 U/ml standard, as indicated in the ELISA data sheet. In preliminary experiments, sCD223 and sCD30 levels were compared in serum and plasma from the same subjects collected at the same time: the ELISA values were comparable.

Statistical analyses

All data were recorded on a computerized database. Results are reported as mean ± standard error (SE). Statistical analyses were carried out using the SPSS software (version 11; SPSS Inc., Chicago, Ill., USA). To study the association between sCD223 and sCD30 and between sCD30 and IgG PT, a linear regression model was applied and the Pearson correlation coefficient was calculated. For comparing overall differences of Th1 and Th2 parameters between the trials and between the positive or negative CMI response, the non-parametric Mann-Whitney U test was performed. Pre/post vaccination differences of Th1 and Th2 parameters within the trials were evaluated by the non-parametric Wilcoxon Paired-Sample Test. *P* values lower than 0.05 were considered to indicate statistical significance and all reported *P* values are two-sided.

RESULTS

sCD223 and sCD30 levels are inversely related

To examine whether there was any relationship between the levels of sCD223 and sCD30, and immune responses to vaccination, we determined both the baseline value of the above factors in pre-vaccinated children and their values after vaccination, irrespective of the type of vaccine administered, i.e, aP-DT or DT. Both sCD30 and sCD223 were indeed measurable, before vaccination, in 2 month-old children. These levels ranged from 27.8 to 187 U/ml and from 0 to14.2 ng/ml, respectively. Similarly, the post-vaccination levels of sCD30 also varied from 32.6 to 153.3 U/ml while that of sCD223 varied from 1.03 to 12.7 ng/ml. Remarkably, there was a clear-cut inverse association in the level of these two markers, both before (r = - 0.99, $P < 0.0001$) and after (r = - 0.95, $P < 0.0001$) vaccination (Fig. 1A, B).

We next compared sCD30 and sCD223 values in aP-DT or DT recipients. We noticed a statistically significant decrease in the sCD30 marker between pre- and post-vaccination levels in DT but not in aP-DT vaccines, suggesting that the presence of pertussis antigens in the vaccine was somewhat instrumental in preventing a fall of sCD30. Overall, in the post-vaccine assay, the highest amount of sCD30, and conversely, the lowest amount of sCD223 were measured in children receiving the pertussis vaccine, with a significant difference as compared with DT vaccine recipients (sCD30: $P = 0.008$; sCD223: $P = 0.015$) (Table I).

Relationship between sCD223 and sCD30 levels and CMI or antibody responses

Since: *i*) not all child recipients of the aP-DT vaccine raised a measurable CMI proliferative response against *B. pertussis* antigens early after post-primary vaccination (2, 3); *ii*) the aP-DT CMI-responsive children demonstrated the prevalence of an antigen-dependent Th2 cytokine profile (3), we next examined whether the production of the above markers was associated with CMI positive response. Interestingly, the population examined appeared to be composed of two groups, one constituted by the 17 CMI responders who showed an increase of sCD30 value (from 88 ± 8.7 to 101.2 ± 5) and the other 16 CMI negative children who showed a decrease of sCD30 (from 98.3 ± 8.2 to 84.2 ± 6.8). The difference in the post-vaccination values of this marker between the two groups is statistically significant (P = 0.034). As expected from the above data, an inverse behavior was shown by the sCD223 marker (Table II). Although no differences here reached statistical significance, it is worth noting that CMI-responsive aP-DT vaccine recipients had the lowest post-vaccination value of sCD223 (7.1 ± 0.6 pg/ml) among all children examined. Overall, the results observed in CMI negative children closely mimicked those seen in DT only recipients (cfs Table II and Table I).

The levels of soluble markers were also examined in relation to the antibody titers against pertussis vaccine antigens (PT, PRN, or FHA). No association was generally found in the levels of sCD30 or sCD223 and the antibody titers, with the exception of a positive association between high sCD30 and high anti-IgG PT antibody titers (Figure 2).

DISCUSSION

The issue of easily measurable serological markers of a protective T cell response in infants receiving primary vaccination warrants attention for monitoring

Table I. *Pre- and post-vaccination levels of sCD30 and sCD223 in children within the Italian pertussis vaccine efficacy trial.*

CMI markers [a]	aP-DT (33) [b]		DT (10)	
	(mean ± SE)		(mean ± SE)	
	pre	post	pre	post
sCD30	93.0 ± 6.0	93.0 ± 4.	79.2 ± 9.9	67.4 ± 7.6(*, **)
sCD223	7.9 ± 0.6	7.9 ± 0.4	9.2 ± 0.9	10.1 ± 0.5(***)

[a] *sCD30 (U/ml) and sCD223 (ng/ml) values were determined by ELISA in the plasma samples obtained before (pre) and after (post) vaccination, as specified in the Materials and Methods section.*
[b] *in parenthesis, the number of children tested.*
The differences between the two groups (aP-DT, DT) of vaccine recipients were assessed by Mann-Whitney U Test.
The pre- post-vaccine differences in the same group of subjects were assessed by Wilcoxon Paired-Sample Test.
**Statistical significance (P = 0.008) relative to post- vaccination sample between aP- DT and DT recipients;*
*** statistical significance (P = 0.028) relative to pre/post- vaccination sample in DT recipients;*
**** statistical significance (P = 0.015) relative to post- vaccination sample between aP-DT and DT recipients.*

the vaccine-induced T cell response. This is of special relevance for vaccines, like the pertussis ones, which, though inducing both high antibody levels and strong T cell responses, have no apparent correlates or surrogates of protection. Thus, chemokines, cytokines and soluble receptors associated with induction of memory T helper responses are primary candidates for the critical roles of these responses in protection. Among these factors, IFN-γ and IL-4/IL-5 would theoretically represent the best tool for tracking Th1/Th2 responses *in vivo*. However, since these cytokines are short-range molecules which are rapidly bound by their receptors and/or inactivated by proteases, their levels in the plasma are not reliable to assess the Th1/Th2 balance *in vivo*. On the other hand, T cell responses in pertussis vaccine recipients can be, and indeed have been, studied

Table II. *sCD30 and sCD223 levels in children participating in the Italian pertussis vaccine efficacy trial: evaluation in children displaying positive or negative CMI responses to PT or PRN antigens.*

CMI markers [a]	aP-DT			
	CMI-positive (17) [b]		CMI-negative (16)	
	pre	post	pre	post
sCD30 (1)	88.0 ± 8.7	101.2 ± 5.0	98.3 ± 8.2	84.2 ± 6.8*
sCD223 (2)	8.4 ± 0.8	7.1 ± 0.6	7.3 ± 0.8	8.7 ± 0.6

[a] *sCD30 (U/ml) and sCD223 (ng/ml) values were determined by ELISA in the plasma samples obtained before (pre) and after (post) vaccination, as specified in the Materials and Methods section.*
[b] *in parenthesis, the number of children tested.*
*The differences between the two groups (CMI-positive and CMI-negative) were assessed by Mann-Whitney U Test:*statistical significance (P = 0.034) relative to post- vaccination sample between positive and negative CMI responders.*

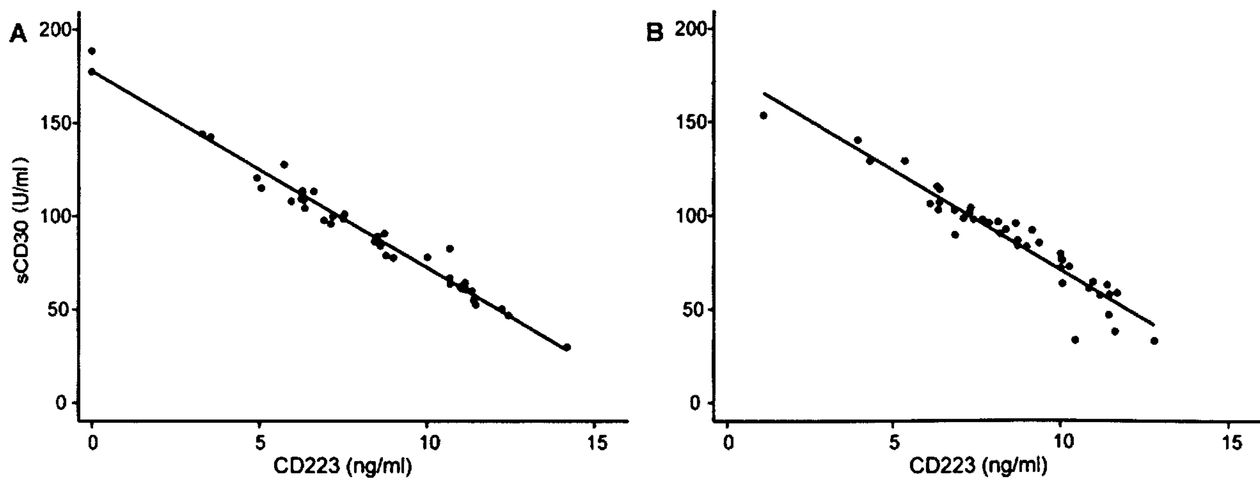

Fig. 1. *Linear regression analysis of sCD30 and sCD223 markers. The values of sCD30 and sCD223 were determined by ELISA, as specified in the Materials and Methods section, in the plasma samples obtained before (panel A) and after (panel B) vaccination, irrespective of the vaccine received. An evident inverse association (panel A: $r = -0.99$, $P < 0.0001$; panel B: $r = -0.95$, $P < 0.0001$) in the level of these two parameters was found.*

with the use of peripheral blood mononuclear cells (3, 6-7, 9-10) but the limitations of sequential blood sampling in very young children are rather obvious.

Along these lines, sCD30 and sCD223 molecules have been considered of particular relevance for their apparent association with Th2 and Th1 polarized response patterns, which are differently stimulated by the different pertussis vaccines (3, 6-7, 9-10). Namely, membrane expression and release of CD30 in soluble form in the blood has been suggested to be associated with preferential activation of Th2 cytokine production, whereas activated Th1 cells express on the membrane, and release the CD223 molecule (24-25, 28). In particular, CD30 expression is restricted to B and T lymphocytes and is dependent on cell type and activation (29-30). The truncated and soluble form of CD30 is preferentially expressed and released by human T-cell clones with a Th2/Th0 phenotype *in vitro* (25). High serum levels of sCD30 have been prevalently found in Th2-dominated disorders, however they have also been found in subjects with rheumatoid arthritis, a disease ascribed to Th1-induced mechanisms (19, 26, 31).

A complex role for CD223 has been proposed (32). The binding of CD223 to its MHC class II ligand on activated T cells negatively regulates T cell function through the CD223/TCR transduction pathway (33). As a recombinant soluble molecule, sCD223 binds with high avidity MHC class II molecules expressed by dendritic cells (DC) (34). This binding leads to DC maturation, migration to the lymph nodes and enhanced cross-presentation of exogenous proteins to the MHC class I pathway (34). Recently, it has been reported that LAG-3 efficiently promotes intra-tumoural recruitment, activation, and Th1 commitment of APCs, and leads to a wide intra-tumoural influx of non-specific and specific reactive cells and to the release of immunoregulatory and cytotoxic mediators (35).

On these premises, the levels of sCD223 and sCD30 in recipients of an acellular pertussis vaccine in the Italian trial of pertussis vaccine immunogenicity and efficacy (2, 14, 36) were assessed. To our knowledge, these soluble potential indicators of distinct Th responses and cellular activation have never been sequentially assessed in children beginning from 2 months of age, and have not been determined in relation to any primary vaccination. In the present study, these soluble factors were measured in sera collected during a double-blind randomized trial, in which the subjects were under tight surveillance for pertussis (and other childhood diseases). During the trial, T cell proliferation, Th1/Th2 cytokines and antibody responses to the various vaccine antigens were assessed longitudinally in vaccine recipients of a sub-population of vaccines (2-3). All this provided us with an important clue for the evaluation of potential relationship between soluble markers of T cell response and vaccine immunogenicity. In addition, the results obtained in the recipients of the aP vaccine were compared with a perfectly matched group of children who were not vaccinated against pertussis but received the DT vaccine only. Last, but not least, the above

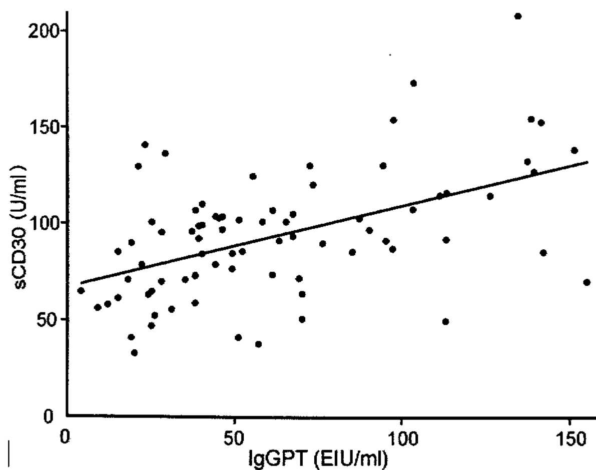

Fig. 2. *Linear regression analysis of sCD30 and anti-IgG PT levels in aP-DT vaccine recipients. The values of sCD30 and anti- IgG PT were determined by ELISA, as specified in the Materials and Methods section, in samples obtained one months after vaccination in 76 recipients of aP-DT vaccine. A positive association was found: r = 0.519, P < 0.001.*

markers could also be measured before vaccination, in children of only two months of age, thus providing the necessary baseline values.

The main results of our investigation can be summarized as follows: *i*) there is a clear-cut inverse relationship between sCD30 and sCD223 in children, both before and after vaccination, strongly suggesting that the two markers are indeed the expression of different and counter-regulated T-cell responses; *ii*) the level of sCD30 decreased significantly from pre- to post-vaccination measurement in children receiving the DT vaccine but not in those receiving the DT plus aP vaccine, suggesting that the presence of pertussis antigens in the vaccine was somewhat inductive of sCD30; *iii*) the increased sCD30 level in the aP-DT vaccine recipients was bound to induction of CMI and anti- IgG PT. In particular, the children who did not acquire a measurable CMI response to pertussis antigens at the post- vaccination assay showed a decrease of sCD30 level, exactly as did the children who received the DT vaccine only.

The inverse correlation between sCD223 and sCD30 has never been previously reported either in vaccinated or unvaccinated subjects. In principle, it would be in accordance with the role assigned to these two parameters as indicators of distinct function of Th cell profiles. Thus, low levels of sCD223 and high levels of sCD30 would be typical of a Th2-oriented response while the inverse would be typical of a Th1 response. A mixed Th0 response would be conceivable with intermediate levels of sCD223/sCD30.

A recent study, where sCD223 and sCD30 blood levels were measured in HIV-negative tuberculosis patients and in healthy household and community controls from Gambia and Guinea, has shown that high levels of sCD223 are present in both healthy exposed contacts and in tuberculosis patients, with favorable outcome. In contrast, the levels of markers of Th2 activity, such as sCD30 and IgE, were high in untreated tuberculosis patients and low in healthy exposed contacts and in patients successfully cured, thus again demonstrating an inverse relationship between the two parameters and their association with Th1 and Th2 oriented responses (37).

Overall, the highest sCD30 and the lowest sCD223 values, indicative of a preferential Th2 pattern, were detected in CMI-responsive children receiving the aP-DT vaccine. These data are well in accordance with the ability of this pertussis vaccine to induce pertussis antigen-specific proliferation and preferentially a Th2 or a Th2/Th0 cytokine pattern in primary vaccination (3, 7), strongly suggesting that modulation of sCD30 and sCD223 markers could indeed be associated with vaccine immunogenicity.

The limitations inherent in the low number of subjects studied and the retrospective nature of this study are to be emphasized here. Nonetheless, our results do clearly invite further, extended investigations aimed at determining the true value of soluble Th markers for estimating the immunogenicity of vaccination in children.

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