Avidity of IgG₁ antibodies to tetanus toxoid: development of an isothiocyanate elution assay and assessment of avidity distribution in a population of healthy children

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Abstract

Dans l'évaluation des réponses vaccinales, la détermination de l'affinité/avidité des anticorps pourrait renseigner sur la maturation des réponses lymphocytaires B, chez des sujets sains ou immunodéficients. L'objectif de ce travail a été de développer un test de mesure de l'avidité des anticorps plasmatiques IgG₁ contre le tétanos toxoïde (TT), basé sur l'utilisation d'un agent chaotropique (isothiocyanate) et de l'appliquer à 90 serums d'enfants en bonne santé. Après optimisation, ce test s'est révélé relativement simple et très fiable. Il a permis de mettre en évidence une augmentation progressive de l'avidité des anticorps anti-TT en fonction de l'âge, avec une corrélation significative entre index d'avidité et log₁₀(âge). Cette augmentation de l'avidité, qui atteint un plateau seulement vers 5 ans, reflète une augmentation progressive du pourcentage des anticorps de haute avidité et une diminution de ceux de faible avidité. Aucune corrélation n'a été relevée entre l'avidité et le taux plasmatique des anticorps anti-TT.

Reference


URN: urn:nbn:ch:unige-1532
DOI: 10.13097/archive-ouverte/unige:153

Available at:
http://archive-ouverte.unige.ch/unige:153

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Avidity of IgG\(_1\) Antibodies to Tetanus Toxoid: 
Development of an Isothiocyanate Elution Assay and Assessment of Avidity 
Distribution in a Population of Healthy Children

Thèse 
présentée à la Faculté de Médecine 
de l’Université de Genève 
pour obtenir le grade de Docteur en Médecine

par

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Thèse n° 10287

Genève 
2002
Table des matières

[Remerciements] .................................................................................................................. 4
[Résumé] .............................................................................................................................. 5
[Introduction en français] .................................................................................................... 6

1 Introduction .................................................................................................................. 11

1.1 Humoral immune response .................................................................................. 11

1.1.1 The antibody – effector function, molecular structure, germline organisation of the immunoglobulin loci ........................................................................ 11

1.1.2 Humoral immune response to thymus(T)-dependent antigens – overview .................................................................................................................. 12

1.1.2.1 Antigen presentation and priming of helper T-cells .................................. 12

1.1.2.2 Activation of B-cells and formation of primary foci ................................ 13

1.1.2.3 Formation of germinal centers ...................................................................... 13

1.1.3 Generation of antibody diversity – the germinal center reaction ..................... 13

1.2 Assessment of the humoral immune response .................................................... 15

1.2.1 Antibody affinity .................................................................................................. 15

1.2.2 Antibody avidity .................................................................................................. 16

1.2.3 Immunoglobulin gene sequence .......................................................................... 17

1.2.4 Biological activity of antibody ............................................................................. 17

1.2.5 Relationships between different qualitative parameters of antibody responses .............................................................................................................. 17

1.3 Maturation of the humoral immune response in early life ................................. 19
2 Material, Methods and Patients ................................................................. 21
  2.1 Material .................................................................................................. 21
  2.2 Methods .................................................................................................. 21
    2.2.1 Determination of serum levels of TT-specific IgG antibodies .......... 21
    2.2.2 Determination of avidity of TT-specific IgG1 serum antibody ......... 22
    2.2.3 Statistical analysis ........................................................................... 22
  2.3 Subjects and serum samples ................................................................. 23
3 Results ...................................................................................................... 24
  3.1 Development and validation of the ammonium isothiocyanate elution assay .. 24
    3.1.1 Introduction ..................................................................................... 24
    3.1.2 Resistance of the coating antigen TT to NH₄SCN ......................... 24
    3.1.3 Duration of NH₄SCN incubation ...................................................... 24
    3.1.4 Dilution of the serum sample .......................................................... 24
    3.1.5 Reproducibility ................................................................................ 25
  3.2 Avidity maturation of TT-specific antibodies in children .................... 25
    3.2.1 Introduction ..................................................................................... 25
    3.2.2 Avidity and age .............................................................................. 25
      3.2.2.1 Avidity index and age ................................................................. 25
      3.2.2.2 High and low avidity fractions and age ...................................... 26
    3.2.3 Avidity and serum levels ................................................................. 26
  3.3 Figures .................................................................................................... 27
4 Discussion .................................................................................................. 34
  4.1 Potentially confounding factors ............................................................. 34
  4.2 Comparison with other reports on the avidity of TT-specific antibodies .... 35
  4.3 Kinetics of avidity maturation of TT-specific antibodies in children ....... 36
  4.4 Avidity testing in clinical medicine ....................................................... 37
5 References .................................................................................................. 39
Remerciements

Mes remerciements les plus chaleureux vont au Professeur Claire-Anne Siegrist et au Docteur Susanna Schlegel-Haueter, sans lesquelles ce travail n’aurait pu être accompli.

Je remercie également Gianna Cadau et Paolo Valenti pour leur aide pratique, le Professeur Paul Henri Lambert pour ses idées précieuses, et le Docteur David Verbel pour son analyse statistique.

Finalement je tiens à remercier le Professeur Susanne Suter de son support et de sa générosité que j’ai beaucoup appréciés.
Résumé

Dans l’évaluation des réponses vaccinales, la détermination de l’affinité/avidité des anticorps pourrait renseigner sur la maturation des réponses lymphocytaires B, chez des sujets sains ou immunodéficients. L’objectif de ce travail a été de développer un test de mesure de l’avidité des anticorps plasmatiques IgG1 contre le tétanos toxoïde (TT), basé sur l’utilisation d’un agent chaotropique (isothiocyanate), et de l’appliquer à 90 serums d’enfants en bonne santé. Après optimisation, ce test s’est révélé relativement simple et très fiable. Il a permis de mettre en évidence une augmentation progressive de l’avidité des anticorps anti-TT en fonction de l’âge, avec une corrélation significative entre index d’avidité et log10(âge). Cette augmentation de l’avidité, qui atteint un plateau seulement vers 5 ans, reflète une augmentation progressive du pourcentage des anticorps de haute avidité et une diminution de ceux de faible avidité. Aucune corrélation n’a été relevée entre l’avidité et le taux plasmatique des anticorps anti-TT.
Introduction

Le système immunitaire défend l'hôte contre des pathogènes tels que des bactéries, des virus, des champignons et des parasites. Quand l’agent infectieux n’est pas éradiqué rapidement par les mécanismes de l’immunité innée, c’est l’immunité adaptive qui entre en jeu avec ses deux composantes, l’immunité cellulaire et l’immunité humorale. Celle-ci agit par le biais des anticorps, des glycoprotéines produites par les lymphocytes B capables de lier les pathogènes (ou leurs produits) de manière hautement spécifique et de neutraliser leurs effets potentiellement nocifs sur l’organisme et/ou de les marquer afin de mettre en route leur destruction. Chaque lymphocyte B produit des anticorps ayant tous la même spécificité pour l’antigène. Puisqu’il existe une immense variabilité de structures au niveau des pathogènes, deux mécanismes principaux augmentent de manière très puissante le rapport variabilité des anticorps/espace utilisé sur le génome pour le codage des anticorps. Le premier mécanisme est celui de la diversité combinatoire, c’est-à-dire une diversification créée par la subdivision des gènes codant pour les anticorps en diverses pièces au niveau du génome, pour chacune desquelles il existe plusieurs variantes et qui, par un mécanisme précisément contrôlé, sont jointes l’une à l’autre par recombinaison au niveau de l’ADN des lymphocytes B, afin que chacun produise un certain type d’anticorps. Ceci a lieu durant le développement des lymphocytes B, c’est-à-dire avant une éventuelle réponse immunitaire à une infection. Le deuxième principe est celui de l’hypermutation somatique des gènes codant pour les anticorps, suivie d’une sélection des cellules produisant les anticorps antigène-spécifiques les plus avides. Ce dernier mécanisme a lieu dans les centres germinatifs des organes lymphoïdes secondaires, rate et ganglions lymphatiques.

Réponse immunitaire humorale

Après l’infection d’un tissu périphérique, l’antigène est transporté vers les tissus lymphoïdes secondaires par des cellules présentatrices d’antigène professionnelles, telles que les cellules dendritiques, où il est présenté aux lymphocytes T et B qui circulent continuellement entre le sang et les tissus lymphoïdes secondaires. L’interaction entre une cellule présentatrice de l’antigène et les lymphocytes T et B spécifiques activent le lymphocyte B qui commencent à proliférer. Après quelques jours de prolifération, une
partie des cellules se différencie en plasmocytes, qui produisent des anticorps à faible affinité, essentiellement du type IgM. D’autres lymphocytes B migrent à l’intérieur de l’organe lymphoïde dans des follicules primaires. Elles continuent de proliférer en formant, avec l’aide de lymphocytes T et en présence de l’antigène, un centre germinatif. C’est dans ces centres germinatifs que surviennent le changement de l’isotype des anticorps produits par un lymphocyte B, ainsi que les mutations somatiques et la sélection des clones B produisant les anticorps ayant des plus hautes affinités pour l’antigène. C’est ce processus, initié dans les centres germinatifs, qui est appelé maturation de l’affinité/avidité. Les cellules B qui émergent de ce processus se développent soit en plasmocytes qui migreront dans la moelle osseuse pour y sécréter des anticorps de haute affinité, soit en cellules B mémoire, qui permettront une réponse immunitaire plus rapide et puissante en cas de réexposition au(x) même(s) antigène(s).

**Affinité et avidité des anticorps**

Une réponse immunitaire humorale contre un certain antigène peut être caractérisée en termes quantitatifs ainsi que qualitatifs. Le paramètre quantitatif le plus fréquemment déterminé est le taux plasmatique d’anticorps spécifiques pour un certain antigène/pathogène. Du côté qualitatif, l’on peut examiner l’isotype, l’affinité, l’avidité, la séquence génétique ainsi que l’activité biologique des anticorps. Ces paramètres sont en partie interdépendants. Par définition, l’affinité d’un anticorps pour un antigène est la force de liaison entre un seul site de liaison de l’anticorps et l’antigène dans une forme monovalente. Expérimentalement elle peut être mesurée par équilibre de dialyse et quantifiée par la constante d’équilibre de la loi d’action de la masse. L’avidité, aussi appelée “affinité fonctionnelle”, est une mesure de la force de liaison entre l’anticorps entier et l’antigène dans sa forme naturelle, qui peut aussi être multivalente et/ou non-soluble. Elle décrit donc plutôt une situation physiologique que théorique ou expérimentale et peut se distinguer clairement de l’affinité, surtout dans le cas d’anticorps IgM avec de multiples sites de liaison et pour un antigène multivalent. Elle se mesure avec un antigène en phase solide, par exemple par test d’élution, un ELISA (Enzyme Linked Immunosorbent Assay) modifié, dans lequel un réactif chaotropique comme l’isothiocyanate (NH₄SCN) est utilisé pour dissocier les complexes antigènes-anticorps.
Puisque les anticorps de faible avidité sont élués par des concentrations plus basses de \( \text{NH}_4\text{SCN} \), la concentration utilisée pour éluer 50% des anticorps liés, appelée « index d’avidité » (AI), est une bonne mesure de l’avidité moyenne des anticorps analysés. Par ailleurs, cette méthode permet des analyses plus élaborées de sous-populations d’anticorps avec des avidités différentes. Une autre technique pour déterminer l’avidité, l’analyse des interactions biomoléculaires (BIA) par résonance plasmonique de surface, est plus performante dans le sens qu’elle permet aussi l’analyse de paramètres kinétiques de la liaison, mais elle ne se prête pas (encore) à l’examen d’une grande série de spécimens, comme il serait nécessaire pour implémer des tests d’avidité en pratique clinique.

**Détermination de l’avidité des anticorps en clinique**

La détermination de l’avidité d’anticorps pourrait devenir un outil clinique d’importance augmentante dans la caractérisation de réponses immunitaires à des vaccinations ou à des infections. Le concept de l’avidité comme mesure de qualité fonctionnelle des anticorps a été démontré dans plusieurs essais biologiques mettant en évidence une corrélation positive entre l’avidité et la capacité protectrice des anticorps spécifiques pour plusieurs germes (par exemple *Haemophilus influenzae b*, *Streptococcus pneumoniae*). Une étude cas-témoin a montré une avidité plus faible des anticorps chez des enfants infectés par *Haemophilus influenzae b* malgré une vaccination antérieure, par rapport à des enfants non infectés. Par ailleurs, la détermination de l’avidité des anticorps a prouvé son utilité dans la distinction entre une infection aiguë et une infection passée (ou non-primaire) par rubéole, toxoplasme, cytomégalovirus ou virus Epstein-Barr, ce qui peut être d’une importance primordiale dans le contexte d’une grossesse ou d’une immunosuppression. Dans la caractérisation des réponses vaccinales, la mesure de l’avidité semble être aussi utile, soit dans la comparaison de différentes nouvelles formules vaccinales (différentes avidités ont été mises en évidence dans des réponses à différents vaccins contre *Streptococcus pneumoniae* et *Haemophilus influenzae*), soit pour évaluer le succès de l’induction de la mémoire immunologique, ce qui peut être spécialement important chez des patients à risque infectieux élevé, comme par exemple après une transplantation médullaire. D’autres applications possibles de la détermination
de l’avidité des anticorps pourraient émerger dans le futur, comme par exemple dans la caractérisation de déficiences immunitaires. Une étude récente a par exemple incriminé une déficience dans la maturation de l’avidité dans un sous-groupe de patients souffrant d’immunodéficience commune variable (CIVD).

**Maturation de l’avidité chez l’enfant**

Dans l’enfance, il y a une maturation de la réponse humorale dans le sens quantitatif ainsi que qualitatif. Les taux d’anticorps de type IgG générés par des vaccinations contre des antigènes T-dépendants sont beaucoup plus bas chez des nouveau-nés par rapport aux enfants plus âgés et aux adultes. Par exemple, même les vaccins les plus immunogéniques chez les nourrissons, tels que *Haemophilus influenzae b* ou tétanos toxoïde, ne parviennent pas à induire des réponses anticorps significatives chez les nouveau-nés, des réponses progressivement plus importantes en terme de taux d’anticorps étant générées lorsque les enfants sont vaccinés à l’âge de 2-3, 4-6, ou 8-10 mois. Peu de données sont encore disponibles concernant la différence de qualité des anticorps induits chez des jeunes enfants et des adultes. Alors que le mécanisme de l’hypermutation somatique semble fonctionnel déjà à la naissance, la maturité de la réponse d’anticorps, en tant que fréquence des mutations et sélection des lymphocytes B produisant les anticorps les plus avides, ne semble atteinte qu’après plusieurs mois, voire plusieurs années. Quelques études ont examiné les réponses à des vaccins polysaccharidiques conjugées, démontrant une maturation d’avidité durant les quelques mois suivant une vaccination. Par contre, à notre connaissance, il n’a jamais été investigué de manière approfondie si cette augmentation progressive de la fréquence des mutations somatiques au cours de l’enfance se traduit directement en une avidité croissante des anticorps induits par un antigène protéique, ni la durée nécessaire à cette maturation d’avidité.

**L’objectif de ce travail a donc été de développer un test d’élution permettant de mesurer l’avidité des anticorps IgG₁ contre un antigène vaccinal utilisé de manière fréquente, à savoir le tétanos toxoïde (TT), dans des échantillons de sérum disponibles en petits volumes (microméthode), permettant l’évaluation de facteurs**
influençant la maturation de l’avidité des anticorps chez des sujets sains ou malades. Après l’optimisation du test, il a été utilisé pour déterminer les valeurs de l’avidité des anticorps anti-TT dans des serums de 90 enfants en bonne santé apparente, âgés entre 5 mois et 15 ans.
1 Introduction

1.1 Humoral immune response

The immune system (Roitt 1998, Goldsby 2000, Janeway 2001) defends the host against infection by pathogens (i.e. bacteria, viruses, fungi, parasites). The first line of defense is provided by innate immunity, which prevents most infections or clears them at an early stage, usually within hours or a few days. Innate immunity generally functions by means of anatomical barriers or by molecular and cellular responses via receptors which recognize features common to many pathogens. Examples for such mechanisms are epithelial surfaces, interferons, the complement system, tissue macrophages, neutrophils, and natural killer cells. However, given the broad variability of pathogen structures and invading or replicating mechanisms, innate immune mechanisms are not always capable of clearing infectious foci, and this is where the adaptive immune response comes in as a second line of defense. The characteristic feature of the adaptive immune response is specificity of its effector mechanisms, which is a consequence of somatic hypermutation of genes encoding for receptor and effector molecules. Somatic hypermutation, a highly controlled mechanism, which may be activated in selected cells during the whole life of the host, permits generation of a much larger degree of diversity of immune response mechanisms than would be predicted from the size of the genome. This contributes to an optimal degree of adaptation of the immune response to a particular pathogen.

The adaptive immune response consists of two lines, the cellular response, mediated through cytotoxic T-cells and type 1 helper T(Th1)-cells, which is directed mainly against intracellular pathogens, and the humoral response, mediated through B-cells, often with the help of type 2 helper T(Th2)-cells, directed mainly against extracellular pathogens. The principal effector molecules of the humoral response are antibodies (also called immunoglobulins), which help other effectors of the immune system to clear the pathogen by specifically binding to its surface.

1.1.1 The antibody – effector function, molecular structure, germline organisation of the immunoglobulin loci

Antibodies exist in two forms: B-cell membrane associated antibodies (i.e. B-cell receptors) are important in the activation of a B-cell to produce antibodies and to increase their avidity. Soluble antibodies on the other hand essentially have two known functions: Neutralization of the pathogen (e.g. by blocking a pathogen-host cell interaction necessary for infection of the latter) respectively of its product (e.g. a toxin), and opsonisation (i.e. coating of the pathogen, which facilitates ingestion and subsequent destruction of the pathogen by phagocytes, and further opsonisation or direct destruction in addition to perpetuation of the inflammatory response by the complement system).

Antibodies are roughly Y-shaped glycoprotein molecules consisting of about equal-sized portions connected by a flexible hinge. The two extremities of the arms of the Y are called V (variable) regions. Their function is antigen binding, and they differ considerably between different antibody populations. The proximal parts of the Y arms as
well as its trunk are called the C (constant) region. It is much less variable, and its function is to activate effector cells and molecules. Antibodies consist of four covalently linked polypeptide chains: Two identical H (heavy) chains, whose amino-terminal parts consisting of 100 – 110 amino acids belong to the V regions and whose carboxy-terminal parts of approximately 330 or 440 (depending on the isotype [see below]) amino acids make part of the C region, and two identical L (light) chains consisting each of an amino-terminal of 100 – 110 amino acids belonging to the V region and of a carboxy-terminal of approximately the same length belonging to the C region. There are several types of C regions determining the isotype (or class) of the antibody (IgG [with subclasses IgG₁, IgG₂, IgG₃, and IgG₄], IgA [IgA₁, IgA₂], IgM, IgD, IgE). The isotype produced by a B-cell depends among other things on the stage of the immune response and the nature of the antigen. For example, early in the immune response after a first encounter with a pathogen, IgM are frequently produced, whereas later on there may be a switch (i.e. isotype switch) to other isotypes, e.g. to IgG subclasses. Different isotypes display different effector functions (e.g. complement is only activated by IgM and selected IgG isotypes), and whereas IgG, IgD and IgE exist in monomeric form, IgA are often covalently coupled to a dimer, and IgM to a pentamer.

There are 3 different gene segments coding for 3 parts of a heavy chain’s V region: the V (variable) segment, the D (diversity) segment, and the J (joining) segment. The C segment codes for the C region. The heavy chain locus on the human chromosome 14 consists of approximately 65 different functioning V gene segments, 27 D segments, and 6 J segments. Moreover it carries one C segment for each possible isotype. Light chain loci are on chromosomes 2 and 22. For light chain V regions, corresponding functional segment numbers are slightly smaller and there are no D segments at all. Otherwise the principle is the same.

1.1.2 Humoral immune response to thymus(T)-dependent antigens – overview

1.1.2.1 Antigen presentation and priming of helper T-cells

At the site of infection, the antigen is captured by professional antigen presenting cells. In the periphery of the body, these are mostly dendritic cells, whereas in lymphoid tissues for example they may also be macrophages or B-cells. The antigen presenting cells then migrate from the site of infection to lymphoid tissues (e.g. lymph nodes, spleen, Peyer’s patches, tonsils). Antigen is processed and presented by major histocompatibility complex (MHC) class 2 molecules to naive helper T-cells, whose T-cell receptors specifically recognize the antigen:MHC complex. Other, co-stimulatory signals are necessary as well. This priming of T-cells takes place in the T-cell zones of lymph nodes and other secondary lymphoid tissues, and the T-cell becomes an armed helper T-cell. Whereas non-activated naïve T-cells continuously circulate between blood circulation and lymphoid tissues, armed helper T-cells are trapped in the T-cell zone of lymphoid tissues.
1.1.2.2 Activation of B-cells and formation of primary foci

B-cells circulate between blood and lymphoid tissues as do T-cells. A B-cell enters a lymph node through specialized high endothelial venules. It then normally moves through the T-cell zone into the B-cell zone (i.e. the primary follicle). However, a B-cell carrying a receptor specific for an antigen, which is present in the lymphoid tissue and to which specific primed T-cells are trapped, is trapped as well in the T-cell zone. Through an interaction between the B-cell and a T-cell, the B-cell gets activated and undergoes several rounds of proliferation. Thereby, the primary focus of clonal expansion is established. The interaction between the cells happens as follows: The B-cell takes up antigen with its B-cell receptors (i.e. the membrane-bound immunoglobulins). Antigen is processed and presented on the cell surface on MHC class 2 molecules. A T-cell specific for a such presented antigen recognizes the MHC:antigen complex with its T-cell receptor. Moreover, a second signal is generated by contact of the CD40 molecule on the B-cell with the CD40 ligand (CD40L) on the T-cell. The activated armed helper T-cell now secretes IL-4 and other cytokines in order to further stimulate the B-cell.

1.1.2.3 Formation of germinal centers

After several days of proliferation in the primary focus, a B-cell can have different fates. Part of the cells undergo apoptosis, others become plasma cells secreting low-affinity IgM antibodies, and some cells migrate into a primary lymphoid follicle to continue proliferation and to form a germinal center together with a smaller amount of antigen-specific T-cells (Berek 1992, Berek 1999). The germinal center establishes a more efficient later response, designed to control longer lasting infections or re-infections. Affinity maturation (i.e. somatic hypermutation and selection) and isotype switching happen at that moment. B-cells undergo several cycles of proliferation and positive selection. Non-selected B-cells die through apoptosis. In order to maintain the germinal center reaction, T-cell help as well as the presence of antigen are believed to be necessary. Antigen is stored in immune complexes on the surface of follicular dendritic cells. It is also possible that a certain antigen level is maintained by proliferation of pathogens within lymphoid tissue. It is thought that B-cells compete for antigen, which eventually remains available for highest affinity cells only. Whereas a germinal center has been initially built by numerous B-cells of several specificities, eventually only a few B-cell clones with the highest avidities survive. B-cells produced and selected during the germinal center reaction will either become high-affinity antibody secreting plasma cells and migrate to the bone marrow, or they may develop into memory B-cells (Meffre 2001), which do not primarily produce antibodies, but which keep the changes they had undergone during the germinal center reaction in order to make a more efficient secondary immune response possible. The molecular mechanisms of germinal center formation are not completely elucidated. Cytokines such as tumor necrosis factor alpha, lymphotoxins as well as their receptors seem to be indispensable (Berek 1999).

1.1.3 Generation of antibody diversity – the germinal center reaction

Antibody diversity is generated by the following mechanisms: 1) Combinatorial diversity (i.e. coupling together of different V, D, and J segments; coupling together of different heavy and light chains; isotype switching), 2) junctional diversity (i.e.
“imprecise” joining of the different segments, so that nucleic acids may randomly be deleted or added), and 3) somatic hypermutation (see below). Joining together of the V region segments, as well as junctional diversity and somatic hypermutation happen at DNA level and are called somatic recombination. Isotype switching happens at RNA level through alternative splicing.

In B-cells, somatic hypermutation leads to diversification of the V region genes (Weigert 1970, Bernard 1978). This mechanism introduces point mutations at a high rate (i.e. 1/1000 base pairs per cell division cycle) into the rearranged genes coding for V regions of heavy and light immunoglobulin chains, so that approximately on every division one nucleotide change is introduced (Nossal 1992). On the amino acid sequence level, such mutations may be silent (i.e. no change), neutral (the change does not alter the function of the antibody), negative (the antibody becomes non-functional or binding strength is diminished), or positive (binding strength is increased). During an immune response to an antigen, B-cells with positive changes will be selected and further stimulated to enter a new proliferation and hypermutation/selection cycle, or to leave the germinal center in order to become an antibody secreting plasma cell or a memory B-cell, whereas other B-cells will undergo apoptosis. Thus, affinity maturation would not be possible without a strict selection mechanism, as otherwise production of B-cells would be too big. For instance, the importance of apoptosis in affinity maturation was assessed by transgenic induction of overexpression of the apoptosis inhibitor Bcl-xL in B-cells. The subsequent antibody response to a specific hapten showed persistence of clones carrying immunoglobulin gene sequences which were absent in control mice, and the average affinity of serum antibodies was lowered (Takahashi 1999). However, affinity maturation does not continue endlessly. There seems to be a so-called “affinity-ceiling” above which a B-cell would not have any selection advantage anymore, as other parameters, such as diffusion coefficients and time used for signal transduction and endocytosis, would become limiting factors of antibody efficacy (Foote 1995). On a molecular level, such point mutations most often lie within the V region. In proliferating B-cells, non-silent point mutations are mostly clustered within three hypervariable regions called CDR1 to 3 (complementarity determining regions) (Griffiths 1984, Kaartinen 1983, Kabat 1991), whereas silent mutations are evenly distributed within the whole V region. Thus, selection seems to favor mutations in the CDRs, which might be explained by the fact that the three CDRs together constitute the antigen-binding surface of the immunoglobulin V region. However, analysis of murine antibody:antigen complexes showed that in spite of a concentration of the majority of hypermutations to CDRs, somatic replacements occurred mainly in residues which were not in direct contact with the antigen (Ramirez-Benitez 2001). Whereas replacement of contacting residues seems to have an adverse effect on binding strength, mutations of more distant residues may have a positive impact. The precise mechanism of somatic hypermutation has not been completely elucidated yet. It seems independent of the actual V region sequence (Azuma 1993) and apparently takes place only under stringent conditions of B-cell activation by antigen and interaction with helper T-cells and follicular dendritic cells (see below). It has recently been described to affect a non-immunoglobulin gene sequence as well, i.e. an intron of the BCL-6 gene sequence (Pasqualucci 1998).

Whereas the B-cell undergoes gene segment rearrangements in central lymphoid tissue (i.e. bone marrow) before antibody production, somatic hypermutation as well as
isotype switching occur for the first time during the second phase of the primary immune response in germinal centers of the secondary lymphoid organs (e.g. lymph nodes). However, affinity maturation can continue through secondary and later immune responses (Berek 1999).

1.2 Assessment of the humoral immune response

An antibody response to infection or vaccination can be characterized in terms of quantity as well as quality. Quantitative measures are serum antibody levels and frequencies of B-cells specific for a certain antigen. Determination of presence and levels of specific antibodies in serum, often done by enzyme linked immunosorbent assay (ELISA), can give answers to clinical questions, such as presence or absence of infection, stage of infection, or presence or absence of protective antibody levels against a pathogen or a toxin. However, not only quantity but also quality of circulating antibodies may be of importance for the efficacy of prevention or clearing of infection. Qualitative parameters characterizing an antibody response are antibody isotype, affinity, avidity, immunoglobulin gene sequence, and biological activity. These parameters are interrelated, i.e. they may be correlated (discussed below).

1.2.1 Antibody affinity

Antibody affinity is defined as the strength of binding between a single antigen binding site of the antibody and a monovalent antigen (Eisen 1964, Siskind 1965, Siskind 1969, Steward 1983, Goldblatt 1997). The antibody/antigen interaction in a homogenous solution in equilibrium can be described with the law of mass action equation: $K_a = k_a/k_d = 1/K_d = [\text{Ab:Ag}]/[\text{Ab}][\text{Ag}]$, where $K_a$ is the association equilibrium constant, $K_d$ the dissociation equilibrium constant, $k_a$ the association rate constant, $k_d$ the dissociation rate constant, [Ab] the concentration of free antibody, [Ag] the concentration of free antigen, and [Ab:Ag] the concentration of bound antibody:antigen complexes. Affinity is typically expressed by either the association or the dissociation equilibrium constant. A high association respectively a low dissociation equilibrium constant indicate a high affinity.

Experimentally, antibody affinity is determined by equilibrium dialysis: A dialysis chamber is separated into two parts by a semipermeable membrane, which is only permeable for the antigen. A known amount of antibody is placed into one part of the chamber, and a known amount of antigen into the other part. Antigen then diffuses across the membrane, and at equilibrium, free antigen concentration is measured. The quantity of bound antigen can then be calculated, and $K_d$ can then be determined mathematically or graphically by performing a Scatchard analysis. For solutions containing a mixture of antibodies with different affinities, an average equilibrium constant can be determined. Another method to determine antibody affinity is fluorescence quenching, where antibody binding aborts fluorescence of previously labelled antigen. Antibody concentration needed to reduce fluorescence to a certain degree can therefore be taken as a measure of affinity.
1.2.2 Antibody avidity

The affinity constant describes the strength of interaction between single antigen binding sites with independent binding behavior on one hand and soluble monovalent antigens (haptens) on the other hand in solutions containing pure preparations of the two components. However, this corresponds rather to experimental conditions than to physiological settings. In reality antibodies are multivalent, containing between 2 (IgG) and 10 (IgM) antigen binding sites, and antigens as well are often multivalent and/or nonsoluble. For thermodynamic reasons, total binding strength is strongly influenced by the number of binding sites, and moreover, binding at one site may induce conformational changes in the antibody and/or the antigen, thus influencing binding at the other site(s) (Goldblatt 1997). Therefore the concept of avidity, or functional affinity, has been introduced (Karush 1970, Karush 1976). Antibody avidity is defined as the overall binding strength of an antibody to an antigen. (However, in literature, the term affinity is sometimes used as well to describe avidity.)

Avidity is measured by two categories of solid phase assays: a) competitive inhibition assays, and b) elution assays (Pullen 1986, Goldblatt 1997). In both of them the antigen is attached to a solid support. In the competitive inhibition assay, antibody is added in solution at a fixed concentration, together with free antigen in different concentrations, and the amount of antigen which inhibits solid phase binding by 50% is determined. The less antigen needed, the stronger the avidity. In elution assays, the antibody is added in solution. After obtaining a state of equilibrium, a chaotrope or denaturant agent (e.g. isothiocyanate, urea, or diethylamine) is added in different concentrations to disrupt antibody/antigen interactions. The amount of antibody resisting elution is determined thereafter with an ELISA. The higher the avidity, the more chaotropic agent is needed to elute a certain amount of antibody. The relative avidity of a heterogeneous mixture of antibodies can be expressed as the avidity index (AI), equal to the concentration of eluting agent needed to elute 50% of the bound antibody. Refined analysis of data can be performed by determining percentages of eluted antibody at different concentrations of the eluting agent. Whereas avidity assays probably describe the reality of antibody/antigen binding better than affinity assays, they still represent an artificial system. The AI is not an absolute but only a relative measure, and for identical pairs of antibody/antigen, it strongly varies as a function of the precise conditions of the assay. Moreover, the coating antigen may be different from antigen found in physiological conditions, i.e. with respect to density and conformation (Goldblatt 1997). Information on low-avidity antibodies might be lost, as on reaching the equilibrium before addition of the eluting agent, lower proportions of low-avidity antibody are bound to the coating antigen than of higher-avidity antibody, which means that higher-avidity antibodies are preferentially detected and analyzed (Butler 2000).

A more recent technology to assess antibody/antigen interaction is binding interaction analysis (BIA) based on surface plasmon technique (Malmqvist 1993). Aside from measuring binding strength, it permits real time determination of binding kinetics, such as association and dissociation rate constants. A further advantage of this highly sensitive method is that labelling of any of the interactants is not necessary. A sensor chip, consisting of a glass slide coated with a thin metal film, to which a surface matrix is covalently attached, is coated with one of the interactants, for example with antigen. A
solution containing the other interactant (e.g. the antibody) is allowed to flow over its surface. A continuous light beam is directed against the other side of the surface, and its reflection angle is measured. On binding of antibody to the antigen, the resonance angle of the light beam changes (as it depends on the refractive index of the medium close to the reactive side of the sensor, which in turn is directly correlated to the concentration of dissolved material in the medium). It is subsequently analyzed with the aid of a computer. Unfortunately, this sophisticated approach would not allow determination of a large number of small volume samples.

1.2.3 Immunoglobulin gene sequence

Immunoglobulin genes of antigen specific B-cells can be analyzed by amplification of nucleic acids by polymerase chain reaction (PCR) and subsequent determination of the gene sequence (Weigert 1970). This permits detection and characterisation of subtle molecular differences between antibody populations which may not always be detected on affinity testing. However, it provides no indication on the influence, either positive or negative, of mutations on antibody affinity.

1.2.4 Biological activity of antibody

Biological activity of antibodies can be assessed by various protocols. For instance, complement-mediated bactericidal antibody activity can be measured in vitro by incubating bacteria together with antibody and complement factors. Subsequent culturing of the remaining bacteria permits to measure the quantity of antibody needed to kill 50% of the bacteria (Weinberg 1986). Similarly, virus neutralisation capacity of antibodies can be assessed by incubating virus particles with neutralizing antibodies followed by transfer onto host cell monolayers. After a further incubation step, numbers of infected cells can be estimated by counting plaques or by detecting viral antigen expressed on host cells (Kalinke 2000). Both approaches are of course limited to pathogens which can be grown in vitro. Methods to assess in vivo activity of antibodies include administration of pathogen-specific immunoglobulins to animals which are subsequently infected with the corresponding pathogen. Titration of the minimal antibody dose to prevent a certain endpoint (e.g. death, presence of pathogen above a certain level in selected tissues, etc.) generates a value of biological significance (Bachmann 1997, Usinger 1999).

1.2.5 Relationships between different qualitative parameters of antibody responses

Affinity and avidity results are not always correlated. For example, early in a primary immune response the predominant antibody isotype is IgM. These antibodies can be produced and secreted before somatic antibody hypermutation of selected clones has taken place, which helps to gain time at the expense of affinity. However, as IgM molecules contain 10 antigen binding sites, the binding to a multivalent antigen increases thermodynamic stability of the complex and thus avidity (Steward 1983). In other cases however, a positive correlation can be found, affinity ranking of murine monoclonal antibodies to dinitrophenyl coupled to bovine serum albumine by equilibrium dialysis gave almost the same results as an ammonium thiocyanate elution assay (Macdonald 1988).
There are many examples for a correlation between affinity and somatic mutations of immunoglobulin variable regions. For example, mice were immunized with the carrier protein coupled hapten 2-phenyloxazolone and the immune response was analyzed. Whereas almost no mutations could be found one week after immunisation, many mutations could be detected another week later which were significantly more frequent in the complementarity determining regions. In parallel affinity increased about 10 times (Griffiths 1984).

Examples for a correlation between antibody avidity and protective capacity assessed in biological assays clearly exist as well. In infants immunized with \textit{Haemophilus influenzae} type b conjugate vaccines, complement-mediated bactericidal serum activity was shown to be correlated with antibody avidity (Schlesinger 1992). Avidity of antibodies to Pneumococcal polysaccharides in adults was found to be correlated with opsonaphagocytic activity and with mouse-protective activity (Usinger 1999). Another group found a tendency to a negative correlation between concentration of Pneumococcal polysaccharide specific antibodies needed for killing of bacteria and avidity in infants (Anttila 1999a). Direct assessment of the significance of antibody avidity for protection against infection in humans is difficult to conduct, however there is evidence for a positive role of avidity. A small case-control study for example found IgG\textsubscript{2} avidity decreased in children who had experienced \textit{Haemophilus influenzae} type b vaccine failure (Breukels 2002).

However, there is also data which supports somewhat less the concepts of antibody avidity maturation and of a positive correlation between avidity and protective capacity in an immune response. Mice were repeatedly inoculated with vesicular stomatitis virus (VSV), a cytopathic virus closely related to rabies virus, which is cleared by a neutralizing antibody response. Antibodies produced after a primary infection at 6 days after inoculation, after secondary infection at 12 days, or after hyperimmunisation at 150 days were found to be of high avidity without any evidence of affinity maturation (Roost 1995). When some of these antibodies were tested \textit{in vitro} and \textit{in vivo} in severe combined immunodeficient (SCID) mice, there was a strong positive correlation between avidity and \textit{in vitro} neutralizing capacity. However, above a minimal avidity threshold, no correlation could be found between \textit{in vivo} minimal protective serum concentration and avidity or \textit{in vivo} neutralizing capacity (Bachmann 1997). The same group then tested recombinant single-chain antibody fragments, consisting basically of a single V region, of one antibody found during the secondary response and detected to be devoid of hypermutations, as well as single-chain antibody fragments which additionally incorporated one or more frequently found hypermutations. They found one hypermutated antibody fragment of 300-fold increased affinity compared to the germ-line precursor. However, when these single-chain fragments were crosslinked with immunoglobulins, imitating polyvalent binding of an antibody molecule, the avidity of the germ-line precursor strongly increased, whereas the binding capacities of the hypermutated fragments did not further improve, which reduced the binding difference to a factor of 10 to 15, instead of 300. Moreover, the several non-hypermutated antibodies showed \textit{in vivo} protective capacities in concentrations which can be expected during an early immune response (Kalinke 2000). Thus, even if it cannot fully be excluded that avidity maturation might have been missed when investigating only after day 6 (Foote 1995), the authors speculate that the phenomenon of affinity maturation may be of less
importance in pathogens cleared by early neutralizing antibodies than in the artificial situation of haptens, where most of the affinity maturation studies have been conducted. These contradictory observations highlight the influence of both the nature of the antigen and the methods used for evaluation, a priori rendering difficult generalization of experimental observations.

1.3 Maturation of the humoral immune response in early life

The level of IgG antibody responses to T-dependent antigens that can be generated in human infants is much lower than that achieved in older children and adults. For example, even the most immunogenic infant vaccines, such as *Haemophilus influenzae type b* or tetanus toxoid vaccines, fail to induce significant antibody responses in >90% neonates (Kurikka 1995, Lieberman 1995), whereas progressively higher serum antibody concentrations are elicited when immunization is given at 2-3, 4-6, or 8-10 months of age (Einhorn 1986, Rowe 2000) Such age-dependent limitations of primary antibody responses are observed with most vaccines (reviewed in Siegrist 2001).

Whereas the quantitative difference in antibody response between infants and adults is well substantiated (serum levels of IgG antibodies, persistence of serum antibodies), there may also be qualitative differences. Qualitative difference between early age and adult antibody responses may concern antibody avidity, but limited data exists on kinetics of avidity maturation to vaccines or infections in infants and children. The relative capacity to induce high-avidity antibodies following immunization in early life has long been questioned. In rodent models, previous analyses have shown that antibody responses to haptens (trinitrophenyl, dinitrophenyl), elicited with corresponding protein conjugates, are characterized by low-affinity antibody-secreting cells following immunization in the first 2 weeks of life (Goidl 1974, Marshall-Clarke 1975, Marshall-Clarke 1978, Doria 1978, Sherr 1979). In humans, it is clear that the mechanisms implicated in avidity maturation, i.e. somatic mutation of immunoglobulin genes and selection of mutated B-cell clones, function already from birth onwards, be it at a quantitatively and maybe qualitatively reduced level. Somatic hypermutation of immunoglobulin genes is present but minimal in cord blood B-cells (Cai 1992, van Es 1992, Nicholson 1995) and present at significant levels at 4 years of age (Klein 1994). Studies performed in infants indicated a progressive increase of somatic mutation of immunoglobulin genes between 2 and 10 months of age, with evidence for selection found from 6 months onwards only (Ridings 1997, Ridings 1998). Avidity maturation was shown to occur following early infant immunization with conjugated polysaccharide vaccines (Goldblatt 1998, Goldblatt 1999, Pichichero 1999, Richmond 2001, Joseph 2001). However, it has been reported that following *Neisseria meningitidis* infection, infants produced specific antibodies of a significantly lower average avidity than older children, which correlated with the absence of bactericidal activity in infant sera (Pollard 2000). Since early protection against infection essentially depends on the presence of pre-existing neutralizing serum antibodies (Ahmed 1996), potential limitations in the capacity to induce high-avidity serum antibodies in early life is of significant concern for early life immunization strategies (Schallert 2002).
Thus, the somatic hypermutation machinery seems to be functional already from the time of birth onwards, but maturity in terms of mutation and selection frequency seems to be achieved after at least several months only. However, whether the increasing frequency of somatic mutations in immunoglobulin genes translates directly into increasing antibody avidity in children, has not been thoroughly examined for protein antigens to our knowledge. The only available data relates to conjugated polysaccharide vaccines, showing evidence of avidity maturation over a period of a few months to 1 year (Goldblatt 1998, Anttila 1998, Pichichero 1999).

The objective of this work was thus to develop an ELISA-based elution assay which could be used to measure the avidity of IgG\textsubscript{1} antibodies to a frequently used vaccine antigen (tetanus toxoid [TT]) in small volume samples, allowing subsequent evaluation of factors affecting avidity maturation in healthy or diseased individuals. Following optimisation, this avidity assay was used to determine the avidity values in sera of 90 immunized apparently healthy children between 5 months and 15 years of age.
2 Material, Methods and Patients

2.1 Material

Flat-bottomed 96 well microtiter plates (Nunc MaxiSorp™) were purchased from Life Technologies, Basel, Switzerland, tetanus toxoid (TT) was obtained from Aventis Pasteur (Lyon, France), Tetuman was from Berna (Bern, Switzerland), biotin-conjugated goat anti-human-IgG antibodies, as well as horse radish peroxidase(HRP)-conjugated extravidine, and 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were obtained from Sigma (St. Louis, MO, USA), HRP-conjugated monoclonal mouse anti-human-IgG₁ antibodies were from Zymed (San Francisco, CA, USA), ammonium isothiocyanate (NH₄SCN) was purchased from Fluka (Buchs SG, Switzerland). All the remaining chemical substances were reagent grade and purchased from Merck (Darmstadt, Germany). The automatic plate washer SkanWash400, the Microplate reader (vmax kinetic) and software (SOFTmax®PRO) were obtained from Molecular Devices Corporation (Sunnyvale, CA, USA).

2.2 Methods

2.2.1 Determination of serum levels of TT-specific IgG antibodies

Serum levels of TT-specific IgG antibodies were determined by ELISA as follows: Microtiter plates were coated with 50 µL/well of TT diluted in carbonate buffer (50 mM, pH 9.6) at a concentration of 5 µg/mL, by incubation over night at 4°C. After 3 washings with 0.9% NaCl containing 0.05% Tween20 using the SkanWash400, the plates were saturated with 100 µL/well of 1% gelatine diluted in phosphate buffered saline (PBS). After blotting the plates on absorbent paper, 50 µL/well of serum samples were added in eight serial dilutions in dilution buffer (PBS containing 0.2% gelatine and 0.05% Tween20), beginning at a dilution of 1:50 and progressing by factor 2. After an incubation at room temperature on a shaker for 30 min and four subsequent washings, biotin-conjugated goat anti-human-IgG antibodies diluted 1:1000 in dilution buffer were added at 50 µL/well. After 30 minutes of incubation at room temperature on a shaker and 5 washings, HRP-conjugated extravidine diluted 1:1000 in dilution buffer were added at 50 µL/well. After further 30 minutes of incubation at room temperature on a shaker and 5 washings, 50 µL/well of freshly prepared substrate solution (0.1% ABTS, 30.5 mM citric acid monohydrate, 44 mM Na₂HPO₄·12H₂O, and 0.01% H₂O₂) were added, and the plates were incubated for 20 minutes in the dark at room temperature. Absorbance was read at 405 nm.

In each plate, a serially diluted standard sample of known antibody concentration (Tetuman) was included, the results were interpolated on the standard by 4-parameter fitting with the SOFTmax®PRO software and expressed in mIU/mL. To test the reproducibility of the method, a positive as well as a negative control sample were included in each plate.
2.2.2 Determination of avidity of TT-specific IgG₁ serum antibody

In order to avoid potential biases resulting from the interference of antibodies of distinct isotypes, we performed our avidity analyses on the main isotype elicited by TT immunization, i.e. IgG₁. An isothiocyanate elution assay based on the method described in the literature (Goldblatt 1997) was adapted in order to measure the avidity of TT-specific IgG₁ antibodies. Coating and saturating of the plate were done as described in 3.2.1. Sera were diluted in dilution buffer, for each serum a dilution factor was chosen to give an optical density (OD) in the upper linear part of the standard curve (OD around 1.5), with a minimal dilution of 1:50. The diluted serum samples (50 µL/well) were incubated for one hour at room temperature on a shaker. After four washings, 100 µL/well of 0, 0.25, 0.5, 1, 2, 3, 4, or 5 M NH₄SCN in dilution buffer were added and the plate was incubated for 15 minutes at room temperature on a shaker. After 4 washing steps by hand, HRP-conjugated monoclonal mouse anti-human-IgG₁ antibodies diluted 1:500 in dilution buffer were added at 50 µL/well. After an incubation of one hour at room temperature on a shaker and 5 washings, substrate solution was added and the absorbance was read as described in 3.2.1.

Each serum was run in duplicate. In each plate, a serially diluted standard sample (Tetuman), to which an anti-TT IgG₁ concentration was arbitrarily assigned, was included without addition of NH₄SCN. The results were interpolated as in 3.2.1 and expressed as mAU/mL of antibody remaining bound to the antigen after the NH₄SCN elution. To test the reproducibility of the NH₄SCN-independent part (i.e. the ELISA part) of the method, a positive as well as a negative control sample were included in each plate. For assessment of the reproducibility of the NH₄SCN-dependent part (i.e. the elution assay), a positive control sample of high avidity was added and run in duplicates.

The avidity index (AI), corresponding to the concentration of NH₄SCN required to elute 50% of the antibody, was calculated as follows: The ODs were transformed into antibody concentrations with the aid of the standard curve (see above). The percentage of eluted antibodies was calculated for each NH₄SCN concentration. These percentages were log transformed, and the two nearest values around log₁₀(50) were chosen. By linear interpolation, the NH₄SCN-concentration corresponding to an antibody concentration of log₁₀(50) was determined. The AI can be seen as a measure of average avidity of a population of antibodies with possibly heterogeneous avidities.

The elution profile was expressed as a histogram (Aviscan) giving the percentages of antibody eluted between two NH₄SCN concentrations (respectively the percentage still adhering at the highest NH₄SCN concentration) plotted against the corresponding concentrations. The Aviscan gives information about the distribution of different avidities within an antibody population of heterogeneous avidities. Arbitrarily, the antibody fraction eluted between 0 and 1 M of NH₄SCN was designed as low avidity antibody fraction, whereas the fraction not eluted at 3 M was designed as high avidity antibody fraction.

2.2.3 Statistical analysis

To examine the relationship of AI (as well as of the percentages of low and of high avidity) of TT-specific IgG₁ antibodies with age as well as with serum levels of TT-
specific IgG antibodies, linear regression using the method of least-squares fitting was performed. Briefly, this is a mathematical procedure for finding the best fitting curve to a given set of observed points by minimizing the offsets (i.e. errors) of the observed points from the theoretical model curve. Inter-observer variability of avidity assay results was tested for a series of AIs, which had been independently measured by two persons. The degree of correspondence between the two sets of values was described by an estimation of Pearson’s correlation coefficient, a measure of linear relationship between two variables. To test intra-observer variability, particularly whether day-to-day variation was greater than variation within one day, covariance parameter estimates were generated. These are measures of the overall deviation of single measured values from a value chosen to minimize offsets. Formal statistical testing was not done due to insufficient sample size. For the same reason, the effect of the degree of serum dilution on variability of the measured avidity could only be tested by simple descriptive summary statistics. All analyses were performed using SAS 8.0.

2.3 Subjects and serum samples

To determine the evolution in avidity of TT-specific IgG₁ antibodies with age, serum samples of children of different ages were tested. Serum samples had been harvested for determination of TT-antibodies to rule out immunodeficiency or define the needs for subsequent vaccine doses. Most children had received 3 TT vaccine doses in the first year of life, followed by a booster in the 2nd year of life and at 4-6 years of age. Samples with TT-specific IgG serum levels less than 1000 mUI/mL, as well as from children with either unknown tetanus immunization history or a previously known or by time of blood testing newly discovered immunodeficiency (e.g. lymphoma, iatrogenic immunosuppression, treatment with intravenous immunoglobulins, etc.) were excluded from the analysis. After reception of the blood, serum had been separated by centrifugation and thereafter stored at –20 °C until analysis. No bleeding was performed for the study, and no other analysis than determination of the quantity and the avidity of TT-specific antibodies was performed.
3 Results

3.1 Development and validation of the ammonium isothiocyanate elution assay

3.1.1 Introduction

To determine the avidity of TT-specific IgG serum antibody, a NH₄SCN elution assay was adapted, based on the general protocol described in the literature (Goldblatt 1997). This is a modified ELISA assay. Between incubation of the antigen-coated wells with antibody-containing serum and addition of the second antibody, it includes an additional step of elution of bound antibodies by the chaotropic agent NH₄SCN. Antibody subpopulations of different avidities are assessed by means of differential elution at incremental concentrations of NH₄SCN and thereafter measuring the corresponding degree of antibody release. Low avidity antibodies are eluted at lower NH₄SCN concentrations, high avidity antibodies at higher NH₄SCN concentrations.

3.1.2 Resistance of the coating antigen TT to NH₄SCN

Testing of resistance of the coating antigen TT to NH₄SCN was assessed by incubating the antigen after coating with NH₄SCN for 15 minutes on a shaker at room temperature. The amount of antigen remaining on the plate was determined by measuring its binding capacity of a known amount of antibody. Up to a NH₄SCN concentration of 3 M, no significant antigen denaturation or elution were observed, whereas above 3 M a limited degree of denaturation or elution was detected (Figure 1). We therefore decided to evaluate elution properties only within an NH₄SCN concentration interval of 0 – 3 M, as at higher concentrations, a reduction of antibody binding could in theory be attributed to antigen denaturation/elution as well as to antibody elution. It should be noted that 3M is already a high NH₄SCN concentration, so that this was not a limitation to further development of the assay.

3.1.3 Duration of NH₄SCN incubation

Varying duration of NH₄SCN incubation showed a strong influence on the results. The longer the incubation, the lower was the AI (AI of control serum depending on NH₄SCN incubation time: 4.0 M at 5 min, 3.8 M at 10 min, 3.5 M at 15 min, 3.4 M at 20 min, 3.1 M at 40 min). We decided in favour of a duration of 15 minutes for all our experiments, as chosen by several authors for this type of assay (Pullen 1986, Goldblatt 1997). This finding illustrates the relative nature of the AI, depending on the conditions of the assay, and therefore the need of standardisation and precise following of a protocol.

3.1.4 Dilution of the serum sample

Variation of serum sample dilution used to assess avidity seemed to have little effect on the results, provided that the measured optical density in the well without NH₄SCN addition was situated in the linear part of the standard curve. In the two samples
tested at four different dilutions each, the AI proved to be very robust, whereas the percentages of high and especially of low avidity antibodies were more variable (Table 1). This is in accordance with the variability of avidity control serum values described below, and therefore this variability seems to reflect inherent properties of the assay itself.

3.1.5 Reproducibility

To test the intra-observer variability of the AIs generated in the assay, a high avidity control serum was included to each plate. In total, 5 test series on different days of 4 to 10 parallel test plates were run. As shown in Table 2, overall degree of reproducibility was very high for AI, high for percentage of high avidity antibodies, and lower for percentage of low avidity antibodies. Weighted average intra-day variability was comparable (data not shown). As expected, day-to-day variation of high avidity control serum AI was greater than within-day variation, as indicated by a covariance parameter estimate for day-to-day variation which was 16 times larger than that for within-day variation (0.002375 vs. 0.00015).

Intra-observer variability was low. 59 different serum samples were tested by two persons each. Reproducibility was high with Pearson’s correlation coefficient estimated at rho = 0.95 (p<0.0001). This illustrates the robustness of the developed assay, allowing its use for evaluation of clinical samples.

3.2 Avidity maturation of TT-specific antibodies in children

3.2.1 Introduction

The primary objective of this study was to describe the avidity maturation of anti-tetanus antibodies in immunized children. This was done by assessing the relationship of avidity parameters (i.e. AI, percentages of low and high avidity fractions of TT-specific IgG₁ antibodies) with age. The secondary objective was to examine the relationship between avidity maturation and serum concentration of anti-tetanus antibodies in this population, i.e. to define whether children raising the highest antibody responses were those raising antibodies of highest avidity, and inversely. This was done by comparing avidity parameters of TT-specific IgG₁ antibodies to the serum concentration of TT-specific total IgG antibodies. For this purpose, avidity of IgG₁ antibodies to TT as well as serum levels of IgG antibodies to TT were measured in serum samples of 90 apparently healthy children of different ages, as described in 3.3.

3.2.2 Avidity and age

3.2.2.1 Avidity index and age

Figure 2 shows the measured AIs as well as the fitted curve, plotted against the age at serum sampling. Overall, the AI is increasing with age. The increase is most marked during the first five years, where the values also seem more heterogeneous than in older children. Later on, a plateau seems to be reached around 3 M. However, as the
values above 3 M were treated as equal to 3 M for reasons of antigen instability at higher
NH₄SCN concentrations, it is possible that the real plateau lies at a higher value. Therefore, the time point when the maturation process reaches completion is not accurately determined by this experiment. Nevertheless, maturation of Al is clearly ongoing until the fifth year of age, which is an unexpected observation. Statistical analysis confirms a significant positive correlation of the Al with age, which can be approximated by the function Al[M] = [2.3324 + 0.6989*\log_{10}(\text{age}[\text{years}])] (p<0.0001). The correlation is stronger between Al and \log_{10}(\text{age}) than between Al and age (not shown).

3.2.2.2 High and low avidity fractions and age

Figure 3 shows the measured high and Figure 4 the low avidity fractions (expressed as percentages of total antibody) together with their respective fitted curve, plotted against age. Whereas the percentage of high avidity antibodies rises from around 30% of total TT-specific IgG₁ to 55% towards the end of the maturation process, the percentage of low avidity antibodies decreases from around 20% to 10% with increasing age. Statistical analysis shows a significant positive correlation of the percentage of high avidity antibody with \log_{10}(\text{age}) (p<0.0001), approximated by the function (% high avidity antibodies) = 30.203 + 23.551*\log_{10}(\text{age}[\text{years}]). Percentage of low avidity antibody was found to be associated with exp(-\text{age}) (p<0.0001), described by the function (% low avidity antibodies) = 9.3503 + 33.876*exp(-\text{age}). Thus, the avidity maturation of TT IgG₁ antibodies is characterized by a progressive increase of high avidity antibodies and decrease of low avidity antibodies.

3.2.3 Avidity and serum levels

Figure 5 shows the Als of TT-specific IgG₁ antibodies plotted against the serum levels of TT-specific total IgG antibodies. No statistically significant correlation was found between these two measured values in our sample of 90 patients (p=0.19). The same applies to the percentages of high (p=0.26) and of low (p=0.32) avidity antibodies in relation to IgG serum levels. Thus, we observed no correlation between the magnitude of TT antibody titers elicited in children and their avidity.
3.3 Figures

![Graph showing resistance of TT antigen to NH₄SCN](image)

**Figure 1.** Antigen resistance to different NH₄SCN concentrations. After coating with TT antigen and subsequent washing, the wells were incubated with NH₄SCN in various concentrations for 15 minutes at room temperature on a shaker. Thereafter, an ELISA measuring the binding of IgG₁ of the standard serum sample to the antigen was done. Each sample was run in duplicate. Results are expressed in % of binding (compared to the samples with 0 M NH₄SCN) as a function of NH₄SCN concentration, and are shown for the serum sample dilution containing a TT-specific IgG₁ concentration of 125 mAU/mL. Results are similar for concentrations between 62.5 and 500 mAU/mL (data not shown).
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<th>High avidity antibody (% of total)</th>
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<td>35.28</td>
</tr>
<tr>
<td></td>
<td>1:400</td>
<td>1.54</td>
<td>4.68</td>
<td>35.15</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>1.48</td>
<td>9.75</td>
<td>38.02</td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td>0.07</td>
<td>4.54</td>
<td>3.45</td>
</tr>
<tr>
<td>CV</td>
<td></td>
<td>4.84</td>
<td>46.58</td>
<td>9.08</td>
</tr>
</tbody>
</table>

*Table 1.* Influence of sample dilution on the avidity parameters. Two serum samples of different avidities were tested in different dilutions, all of which, however, gave ODs in the linear part of the standard curve. Results are given individually and as mean, SD and variation coefficient (CV = (SD/mean) * 100) for each of the samples. Variation was small for the AI and the percentage of high avidity antibodies, whereas it was higher for the percentage of low avidity antibodies.
Table 2. Reproducibility of the avidity assay. A high avidity control serum was included in each of the 36 plates tested on 5 different days. Overall reproducibility degree was high for AI and for percentage of high avidity antibodies, and lower for percentage of low avidity antibodies. Weighted average intra-day variability was comparable (not shown).
Figure 2. Avidity index (AI) of TT-specific IgG_1 antibodies in relation to age. The AIs of 90 serum samples of apparently healthy children were plotted against age. The AI increases with growing age reflecting avidity maturation of TT specific antibodies of the IgG_1 subclass during childhood. The AI is associated with log10(age) (p<0.0001), this association is approximated by the function AI[M] = [2.3324 + 0.6989*log10(age[years])] (shown as a continuous curve). AI values of >3 M were arbitrarily assigned the value of 3 M (already before statistical analysis).
Figure 3. Fraction of high avidity IgG₁ antibodies specific for TT in relation to age. The high avidity fractions (i.e. not eluted at NH₄SCN concentrations up to 3 M) were plotted against age. In accordance with the AI increase with growing age, the high avidity fraction increases as well. The percentage of high avidity antibody is associated with log₁₀(age) (p<0.0001), which is approximated by the equation: (% high avidity antibodies) = 30.203 + 23.551*log₁₀(age[years]).
Figure 4. Fraction of low avidity IgG1 antibodies specific for TT in relation to age. The low avidity fractions (i.e. eluted at NH4SCN concentrations < 1 M) were plotted against age. In accordance with the high avidity fraction increase, the low avidity fraction decreases with growing age. The percentage of low avidity antibody is associated with exp(-age) (p<0.0001). The function approximating this relationship is: (% low avidity antibodies) = 9.3503 + 33.876*exp(-age).
Figure 5. Comparison of the avidity of TT-specific IgG1 antibodies with the serum levels of TT-specific IgG antibodies. No statistically significant correlation was found between antibody avidity and antibody serum levels (p=0.19).
4 Discussion

We adapted an isothiocyanate elution assay previously described and validated for other antigens (Goldblatt 1997) to measure the avidity of IgG\textsubscript{1} antibodies directed against tetanus toxoid (TT) by assessing and - where indicated - optimizing several parameters, such as duration of incubation with NH\textsubscript{4}SCN, resistance of the antigen used for coating to NH\textsubscript{4}SCN, and dilution of serum samples. These optimization/validation steps were all successful, allowing to apply the assay to serum samples of 90 apparently healthy children aged between 5 months and 15 years with known TT immunization history. The objective of this cross-sectional study was to describe avidity maturation by assessing avidity distribution of TT-specific IgG\textsubscript{1} antibodies in children of different ages.

Avidity of IgG\textsubscript{1} TT antibodies was found to increase with age. A direct correlation was observed between avidity index and $\log_{10}(\text{age})$. The avidity increase was most marked during the first five years of life. Whereas for most children younger than 1 year AI was in the range of 1.5 - 2.5 M, for all children aged 4 years and more, the AI lay above 2.5 M. After about 5 years, the AI seems to reach a plateau around 3 M. The rise of the high-avidity antibody fraction (i.e. not eluted at NH\textsubscript{4}SCN concentrations up to 3 M) with increasing age is in accordance with the rising AI. A statistically significant correlation between the percentage of high avidity antibodies and $\log_{10}(\text{age})$ could be detected as well. It also seems to reach a plateau around 5 years. The percentage of low avidity antibodies decreases with age, it was found correlated with $\exp(-\text{age})$. Two samples showed a significantly lower AI than samples of other children of comparable age. One child, displaying an AI of 1.17 at 2.2 years was found to have received an incomplete (i.e. 2 doses) and delayed (i.e. at 11 and 12 months) course of primo-vaccination, which probably explains the low value. For another child with a low AI of 2.1 at 3.8 years, no explanation could be found. No correlation could be found between avidity and TT-specific total IgG levels.

4.1 Potentially confounding factors

We examined serum samples of children who were evaluated to rule out an immunodeficiency, i.e. who may not be fully representative for a population of healthy children. However, the presence of such a deficiency seems highly improbable in children mounting an strong antibody response to TT immunization (titers $>$1000 mUI/ml), so that we considered them as apparently healthy. This assumption is further supported by the fact that the method developed during this thesis project was subsequently used in a longitudinal follow-up study to assess the avidity of TT-specific IgG\textsubscript{1} antibodies in 400 Gambian children at 5 and at 12 months of age. AIs ($1.56 \pm 0.34$ M and $2.28 \pm 0.30$ M at 5 and 12 months of age, respectively) and percentages of high and of low avidity antibodies were comparable with the results obtained in our population (Schlegel-Haueter S et al., manuscript in preparation).

Our avidity assessment was restricted to IgG\textsubscript{1}, whereas the serum levels were assessed for total IgG. Since TT-specific IgG antibodies are mainly of the IgG\textsubscript{1} isotype (Devey 1985, Kroon 1999, Aboud 2001), and since a very close correlation between TT-specific IgG and IgG\textsubscript{1} levels has been observed in our samples (not shown), it can be
safely assumed that the IgG₁ fraction makes up almost the entire IgG population and is therefore representative for the total IgG fraction. The chemical stability of the coating antigen not being guaranteed at NH₄SCN concentrations above 3 M, we decided not to assess AIs above this value. With this restriction some AIs might be underestimated, as some samples, especially of adults (not shown), seem to have AIs slightly above 3 M. Further experiments may be indicated to assess the stability of the antigen at conditions closer to those of our assay, i.e. in presence of serum. In any case, this theoretical concern does not affect the main observation of a very prolonged maturation period, extending over 5 years. The lower limit of TT-specific IgG antibody levels at 1000 mIU/mL as an inclusion criteria, used in this study to rule out immunodeficiency, may probably be unnecessarily high with respect to applicability of the assay. Further experiments would be indicated in order to determine the lower limit above which the assay works reliably. This would allow evaluation of the avidity of TT antibodies in patients with suboptimal or weak TT-specific responses.

4.2 Comparison with other reports on the avidity of TT-specific antibodies

A general problem for the comparison of avidity studies is the plethora of different techniques of measuring avidity and the different definitions of AI and high and low avidities. Some authors only use one fixed concentration of the eluting agent and define the AI as ratio of optical density of the well with and without eluting agent (Abacioglu 1995). Others define the AI as concentration of eluting agent required to reduce the optical density by 50% (Avanzini 1998), which may yield different results than measuring reduction of antibody concentration, as soon as values lie outside the linear part of the standard curve.

A cross-sectional study on levels and avidity of IgG antibodies to TT in 138 Tanzanian children aged 1-15 years reported that in 1-5 year-old children, 89% had a high AI, whereas among 6-15 year-old children only 52% had a high AI. (Aboud 2000). In this study, high and low AIs were defined as a ratio of optical density of the wells with and without eluting agent (i.e. urea) of >50% and <30% respectively. This apparent contradiction to our results, i.e. a reduction of antibody avidity with age, may be explained by several differences in methodology. Whereas we only considered serum samples with TT-specific IgG levels of ≥ 1000 mIU/mL, this group also included samples with antibody levels considered as unprotective, i.e. below 100 mIU/mL, which may rise questions regarding the quality of avidity values apparently not validated at such low serum levels. They indeed found a strong association between low AI and non-protective antibody level in multivariate logistic regression analysis. This might be at least partially explained by unsuccessful priming, administration of insufficient number of vaccine doses, or failure to detect small amounts of high-avidity antibodies. Indeed, their immunization schedule differed from ours in that the third DPT vaccine dose was given already by the 12th week of life, while a booster during childhood was not scheduled in a standardized way. Moreover, for children between 6 and 15 years of age, documentation of immunization was frequently missing. This might as well introduce a certain bias, as e.g. the same group showed in another study in adult Tanzanian women that TT-specific IgG levels were significantly higher in women presenting written documentation of immunization compared to women with oral history of immunization only (Aboud 2001).
A further factor to complicate comparability between the two studies is the application of a different assay (elution assay with urea at one single concentration, different definitions of AI and high avidity [see above]).

TT-specific antibody avidity has been assessed in other contexts as well. One study tested sera of mother-infant pairs at birth and found that TT immunization during pregnancy resulted in higher anti-TT IgG levels without affecting the avidity (Abacioglu 1995). These results are in accordance with the concept of an already matured immune response resulting from previous immunization. The study found no significant differences in AIs within the mother-infant pairs. Another group found significantly higher IgG AIs in infants compared to those of their mothers in a small group of mother-infant pairs. This was independent of putative antigen load, and no correlation was found between antibody level and avidity. Antibody concentrations had a tendency to be higher in infants than in their mothers. The authors postulate a preferential transplacental transfer of high avidity antibodies (Avanzini 1998).

Affinity of TT-specific antibodies was shown to be lower in protein-energy malnourished children than in healthy controls (Chandra 1984). A small study assessing affinity of TT-specific antibodies in freshly immunized adults showed failure of affinity maturation in patients with rheumatoid arthritis, whereas adults with systemic lupus erythematosus and healthy controls showed increase of affinity (Devey 1987). Whereas patients with AIDS showed a reduced quantitative antibody response to TT immunization recall, affinity of TT-specific IgG was found equally high as in controls (Janoff 1991). Similar results were obtained with avidity of TT-specific IgG antibodies (Kroon 1999).

4.3 Kinetics of avidity maturation of TT-specific antibodies in children

Our results show a prolonged avidity maturation period of TT-specific IgG antibodies during childhood. This raises two interesting issues: First, the duration of avidity maturation during an immune response (Berek 1999). As far as antibodies to TT are concerned, its extension over several years of age clearly demonstrates that although germinal centers are needed to initiate somatic hypermutation, B-cell competition and clonal selection, i.e. initiate avidity maturation, this process extends well beyond the few weeks during which germinal centers are observed. The existence of a post-germinal center compartment in which affinity maturation takes place has indeed be postulated in mice (Smith 2000), and our observations suggest that it may exist in humans as well. It will be of interest to try to understand the factors defining whether avidity maturation is essentially completed within a few months (such as following hepatitis B immunization, [Siegrist CA et al., manuscript in preparation]) or extends for several years. Persistence of antigen, either at the site of injection or at the surface of follicular dendritic cells, could play an important role.

The second interesting issue is that of immune system maturation during childhood (Ridings 1997, Ridings 1998, Siegrist 2001). As all of the children in our study were immunized at about the same age, it is not possible to give a clear answer to the question whether the observed rise in avidity was due to the maturation of the immune system during the first years of life or rather to the inherent duration of an avidity maturation of an immune response to a vaccine. Data on avidity maturation of TT-
specific antibody in humans who were primo-vaccinated as adults is scarce. One healthy adult woman showed antibodies of a high AI (>3 M) within the first year after primo-immunisation, suggesting faster avidity maturation than following infant immunization (data not shown). Repeated measurements of anti-rubella antibody avidity in formerly non-immune primo-vaccinated adult women seemed to show an equally fast maturation within weeks (Pullen 1986). Thus, the low average avidity values in young children might reflect the immaturity of their B-cell immune responses rather than a normal, age-independent slow rate of avidity maturation during years. This could be related to factors limiting the development of germinal centers in early life, as recently observed in infant mice (Pihlgren M et al., manuscript submitted). Interestingly, this limitation of germinal center induction in infant mice is associated with a limitation of avidity maturation of antibody responses to small peptides or haptens, whereas responses to more complex protein antigens (pertussis toxin, tetanus toxoid) were similar to those in adults, although requiring several months for maturation (Schallert 2002).

4.4 Avidity testing in clinical medicine

Measuring avidity is more and more appreciated as an important tool in clinical medicine, be it in diagnosis of infection or in assessment of new vaccines or immunisation responses of immunocompromized patients. For instance, in the presence of immunoglobulins, avidity determination was able to distinguish between acute and past (respectively primary and non-primary) infections from rubella (Hedman 1988), toxoplasma (Hedman 1989), cytomegalovirus (Bodeus 1998, Bodeus 1999), or Epstein-Barr virus (Schubert 1998). Such distinctions may be of primordial importance in the context of pregnancy or of immune suppression. Measuring avidity in human immune response can be important for the assessment of novel vaccine formulas, as for example different pneumococcal or Haemophilus influenzae type b conjugate vaccines were found to elicit different avidity responses (Schlesinger 1992, Anttila 1999b, Goldblatt 1998). Avidity can also serve as a surrogate for successful priming by induction of immunological memory. In infants with low Haemophilus influenzae type b-specific antibody following primary immunization with Haemophilus influenzae type b conjugated vaccines, antibody avidity after booster immunization was still low, indicating absence of priming (Goldblatt 1998). Antibody avidity has also been found to be a surrogate marker of priming after meningococcal conjugate vaccines (Joseph 2001). Assessment of priming is particularly important in patients at high risk for complicated infections, such as immunosuppressed patients after a bone marrow transplantation. When immune response to a standard pneumococcal polysaccharide vaccine was assessed in children who were immunized at different time points after bone marrow transplantation, an inadequate induction of serum antibody was not only found in terms of antibody level, but avidity declined significantly in the majority of patients and remained low for months, which seemed to suggest the need of alternative immunisation strategies in children after bone marrow transplantation (Spoulou 2000). Our own preliminary observations of avidity maturation of the immune response to TT in bone marrow transplanted adults seemed to reveal distinct patterns of avidity responses: in some patients high avidity antibodies could be found soon after the primary post-transplantation immunisation, whereas others seemed to undergo an avidity maturation process similar to that of primo-vaccinated children (data not shown). The significance of
this phenomenon is not clear yet, but suggests persistence of memory cells, despite irradiation and chemotherapy, which are readily recalled by administration of a single vaccine dose after transplantation.

A recent paper incriminated an avidity maturation defect in the pathogenesis of a subset of patients with common variable immunodeficiency (CIVD). They found a subset of patients, clinically characterized by a later onset of the disease, which showed significantly lower frequency of somatic mutations in a V gene region of circulating B-cells. Further on, there was neither evidence for an abnormal selection process nor for a quantitative functional defect of the hypermutation machinery (i.e. mutation rate in the non-immunoglobulin BCL-6 gene was not diminished in these patients) and therefore suggested a defect of targeting of the hypermutation machinery as possible characteristic of such patients (Bonhomme 2000). It may thus also be of high interest to investigate the role of avidity maturation in immunodeficiency syndromes and the utility of relatively simple avidity assays as diagnostic tools.
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