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RESEARCH ARTICLE

AFFINITY OF TETANUS VACCINE IN THE LATE PRIMARY IMMUNE RESPONSE AFTER THE END OF THE GERMINAL CENTER REACTION

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Abstract

The immune response of experimental animals against tetanus toxoid (TT) vaccine was developed within 14 days post vaccination. Recorded data revealed that antibody (Ab) level was time related, and the intraperitoneal (IP) administration of vaccine showed a higher Ab level in a significant way than subcutaneous (SC) one. Avidity index showed higher significant difference in case of IP administration than SC one. The avidity index post intravenous administration of anti-CD154 (MR1) monoclonal Ab was detected using ELISA technique and germinal centers (GCs) were detected using immunofluorescence assay (IFA). Histopathological examination revealed that the peaking of GCs was on the 10^{th} day post vaccination and an obvious deterioration was recorded on day 33 post vaccination. It was found that the avidity index of serum Ab in MR1 injected group was significantly lower than non MR1 injected group at day 69, suggesting that GCs generate the precursors of high affinity BM AFCs. This finding suggests that memory B cells and long-lived antibody secreting plasma cells may represent independently regulated cell populations and may play different roles in the maintenance of protective immunity.

Introduction:-

Tetanus toxin, the product of Clostridium tetani, is taken up into terminals of lower motor neurons and transported axonally to the spinal cord and/or brain stem. The toxin moves trans-synaptically into inhibitory nerve terminals, where vesicular release of inhibitory neurotransmitters becomes blocked, leading to dis-inhibition of lower motor neurons **(Hassel, 2013).** The germinal center (GC) reaction is the basis of T-dependent humoral immunity against foreign pathogens and the ultimate expression of the adaptive immune response. GCs represent a unique collaboration between proliferating antigen-specific B cells, T follicular helper cells, and the specialized follicular dendritic cells (FDCs) that constitutively occupy the central follicular zones of secondary lymphoid organs. The primary functions of GCs is to produce long-lived high-affinity antibody-secreting plasma cells and memory B cells that ensure sustained immune protection and rapid recall responses against previously encountered foreign antigens **(Gatto and Brink, 2010).** Somatic hypermutation (SHM) is a process in which point mutations accumulate in the antibody V-regions of both the heavy and light chains, at rates that are about 10^6-fold higher than the background mutation rates observed in other genes **(Zhang et al., 2001 and Bemark et al., 2003),** this accumulation of mutations at the V-region genes occurs at the centroblast stage of B-cell differentiation in the dark zones of GCs in secondary lymphoid organs, whereas the overall goal of this process is to produce high-affinity Abs after selection in the light zone of the GC. The avidity (functional affinity) is an important binding parameter that characterizes the strength of the complex between an antigen (Ag) and an antibody. In contrast to the binding affinity, which is a quantitative parameter, the antibody avidity is a semi-quantitative parameter. The avidity of antibodies is a derivative of the binding affinity as well as of the antibody valency, the density of epitopes, and the antibody

polyreactivity **(Moquet et al., 2010).** Antibody affinity maturation (AAM) referred originally to the observed increase in average Ab affinity against a hapten. Later, it was found that AAM was associated with the formation of transient lymphoid structures in the B cell zones of lymphoid tissues, called germinal centers (GCs), during T-cell dependent immune responses in higher vertebrates **(Flajnik et al., 2002).** Concomitantly, Antibody-forming cells (AFCs) accumulate during the late primary immune response in the bone marrow (BM), As a result, a few months after immunization the great majority of antigen-specific AFCs are present in BM sustaining long-lasting serum Ab titers **(Manz et al., 1997 and Slifka et al., 1998).** Thus, cellular events leading to the preferential accumulation of high affinity AFCs in BM are key elements in the affinity maturation of serum antibody and are crucial for protective immunity. In the present study we show that serum Ab affinity maturation continues to increase long after the end of the GC reaction and it could be blocked significantly by disruption of the GCs before their ending with anti-CD154 (CD40L) antibody (Fig. 1), as GCs are the major sites of generating high affinity BM AFCs, and the BM is considered the major site of long-term Ab production. However, the subsequent selection for higher affinity AFCs in BM does not require GCs, and affinity-based selection among BM AFCs continues long after the end of the GC reaction. The present work aimed to evaluate the anti-tetanus immune sera avidity and the potential persistence of germinal centers post vaccination as a predictive monitoring of the immune response of vaccines which may be at risk of infection and for the assessment of vaccination and vaccination schedule efficacy.

Materials and methods:-

Alum adsorbed TT vaccine was kindly supplied from **VACSERA –Egypt** as 40 IU / ml. It was used to immunizing mice groups. Vaccine was administered as intraperitoneal and subcutaneous route of administration. Immune response against TT vaccine was monitored using homemade ELISA and according to the manufacturer protocol, where plates were coated with purified TT antigen as 2 µgm/ml for 18 hrs at room temperature. Non-adsorbed TT Ag was decanted post washing with wash buffer (phosphate buffer saline(PBS) + 0.05% Tween 20) using Biotek-LX -405 plate washer for three times at 5 minutes intervals. Plates were blocked using blocking buffer [PBS+ 3% BSA]. Immune sera samples were diluted as 1/10 in dilution buffer [PBS+ 1% BSA]. Plates were incubated at 37^{o}C for One hr and washed as previous. Anti-mouse conjugate labeled with peroxidase enzyme was added as 1/1000 final dilution in DB for an hour at 37^{o}C. Plates were washed as previous. Substrate buffer (TMB) Sigma Aldrich was added as 0.1 ml / well. Stop solution [**2N H_2SO_4**] was added. Plates were read at 450 nm using ELISA reader Biotek-ELx-800). Optical density represent antibody levels were plotted against the time interval post vaccination. Avidity was performed according ELISA protocol except addition of NH4SCN concentrations **(Pullen et al., 1986)**. The percentage of Ab molecules which remain bound to the plates at NH4SCN concentration over 1.7M is arbitrarily considered as the higher avidity fraction. BSA and stop solution and peroxidase conjugate were purchased from Sigma- Aldrich-USA.

BALB/c mice:-

Were kindly purchased from VACSERA animal house. Adult Mice were of 18-22 gm body weight, and bred in laboratory animal department in the - **CLAVCAP-VACSERA** at standard condition of day and light and were fed using dry food of 24-26% protein content.

Blood sampling:-

Blood samples were collected from the retro-orbital plexus to evaluate the immune response. Collected blood samples were left to coagulate at room temperature and cold centrifuged at 3000 rpm. Sera samples were harvested in new labeled cryotube and kept at -70^{o}C till evaluation of immune response and avidity of anti-tetanic sera.

Mice Immunization:-

Immunization of mice was conduct using subcutaneous (SC) and intraperitoneal (IP) routes of administration using 16 IU/ ml / mouse as 0.2 ml.

Antimouse conjugate:-

Kindly purchased from (Sigma–Aldrich, USA). It was prepared in goat and labeled with peroxidase enzyme. It was used as 1/1000 final dilution.

MR1 antibody (anti-CD154):-

Kindly purchased from (e-bioscience, USA) and was administered using intravenous injected at days 6 and 8 after IP immunization and at days 48 and 50 after SC and IP immunization with 250 µg.

PNA-FTIC:-

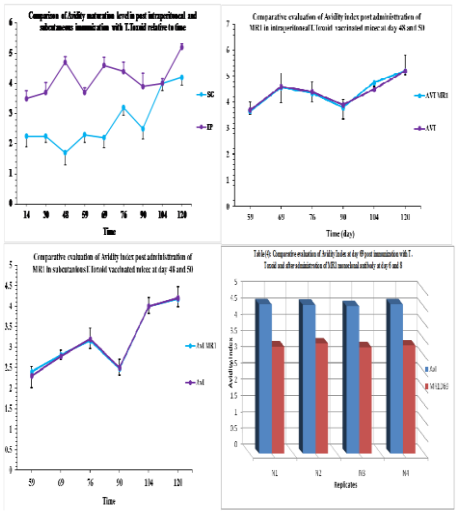
Kindly supplied from (Sigma–Aldrich, USA) and used for detection of GCs in spleen tissues it was used as 1mg/ml.

Immunohistochemistry:-

Spleen tissues from naive and immunized mice were frozen in Tissue-Tek OCT compound (**Sakura, Zouterwoude, The Netherlands**) and cut into 10 µm cryosections at four levels separated by at least 200 µm. Sections were fixed in acetone for 10 min and stored at −20 ˚C till staining. After rehydration in TBS, sections were blocked for 30 min with $\mathbf{H_2O_2}$ in methanol and then blocked for 20 min with 10% normal Rabbit serum for detection of GCs, four sections from each spleen were stained with PNA-FITC (Sigma L7381 2mg/2ml PBS) diluted 1:1000 in Tris/PBS/BSA diluent for 60 min RT. 2x rinse PBS, 5 min PBS bath, 2x rinse PBS and imaged under fluorescent microscope (Nikon, Japan).

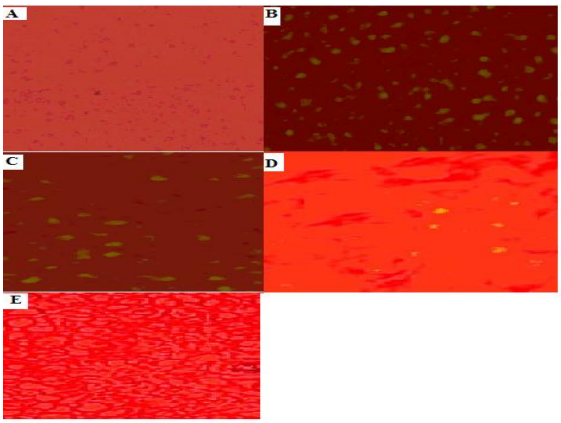
Results:-

Result:- Evaluation of avidity of TT vaccine on the immune response [Fig.1], data revealed that in case of IP and SC immunization of mice there was a highly significant avidity index post immunization ($P<0.05$). In case of MR1 administration at days (48-50) there was a non-significant AVI in mice sera in case of IP and SC administration ($P>0.05$). While in case of MR1 administration at the 6th and 8th days post vaccination the avidity index at day 69 was significantly elevated in case of IP administration of TT vaccine. Regarding the immune Ab titers [Fig.2], antibody levels could be detected on the 14th day post vaccination and there was non-significant Ab levels difference at the 14th and the 120th days post vaccination, while in the in-between duration there was a significant (48, 59); $P<0.05$), and highly significant difference (day 30, 69,76 and 90) ; ($P<0.01$). The Abs levels in mice groups with and without MR1adminstration at day 6 and 8, there was a highly significant difference in Ab levels in non-MR1 administered group than in MR1 administered group ($P<0.01$) on the 69th day post administration. In case of SC group of MR1 administration at days (48-50) post vaccination there was a significant difference in Ab levels at 59th and 69th days and the later on Ab levels were non-significantly related. While there was a non-significant difference in Ab levels post IP administration ($P>0.05$). Histopathological examination revealed the peaking of GC B cells was on the 10th day post vaccination and the least peak was on the 33rd day**,** regarding the duration of GC B cells in splenic tissue it was found that GC B cells existence was time related as its presence was decreasing relative to time in both intraperitoneal and subcutaneous administration of TT vaccine [Fig.3].



[Fig.1] Evaluation of Avidity index in sera samples of TT vaccinated mice using Sandwich ELISA post intraperitoneal and subcutaneous vaccination

[Fig. 2] Evaluationof antibody titer post immunization of mice with alum adsorbed TT vaccine both intraperetoneal and subcutaneous vaccination using Sandwich ELISA



Detection of GC in splenic tissues post Intraperitoneal and subcutanious adminstration of TT vaccine using IFA A: None immunized mice , B : GCs post IP vaccinationat day 10. C: GCs post subcutanious vaccination at day 10 , D: GCs cells atday 33 day post intraperitopneal vaccination with TT vaccine and E: GCs cells at day33 day post subcutanious vaccination with TT vaccine

Discussion:-

The goal of vaccination is to induce long-lasting protective immune memory. Although most vaccines induce good memory responses, the type of memory induced by different vaccines may be considerably different. In addition, memory responses to the same vaccine may be influenced by age, environmental and genetic factors. Results emerging from detailed and integrated profiling of immune responses to natural infection or vaccination suggest that the type and duration of immune memory are largely determined by the magnitude and complexity of innate immune signals that imprint the acquired immune primary responses **(Flora Castellino et al. 2009)**. Antibody affinity maturation is a well known process that characterizes immune responses to T-cell dependent antigens; the process takes place primarily in well known transient microenvironmental structures known as germinal centers (GCs) which are formed in secondary lymphoid organs in response to antigenic stimulation. In GCs, somatic hypermutation (SHM) of antibody V- region genes is initiated and produces multiple antibody variants which are selected according to their affinity for the Ag which is sequestered on FDCs **(Jacob et al., 1993)**. Lower affinity, autoreactive and non-specific GC B cells are negatively selected and undergo default mechanism apoptosis while GC B cells with increased affinity for Ag get additional co-stimulatory signals and survival from Ag-specific follicular T cells (TFHs) leading to further hypermutation and selection and/or production of long-lived BM plasma cells and memory B cells sustaining long-term Ab production **(Tarlinton, D.M., 2008)**. The avidity (functional affinity) is an important binding parameter that characterizes the strength of the complex between an Ag and an Ab. In contrast to the intrinsic affinity (the strength of one binding site), the avidity is the summation of all binding sites of specific serum Abs which represents the real binding parameter in vivo and is important in maintaining protection against infectious pathogens including toxins like tetanus and diphtheria and low avidity Abs was accompanied with defective protection **(Romero-Steiner S. et al, 1999)**. With regards to the long-term production of antibody, two hypotheses have been proposed to explain the longevity of the antibody response in the absence of re-exposure to antigen. First, it has been suggested that antibody levels are maintained by the presence of long-lived plasma cells in the bone marrow, secreting specific antibody for extended periods, possibly several years **(Manz et al., 1997 and Slifka et al., 1998).** The second hypothesis suggests that memory B cells are continually differentiating into plasma cells in an antigen-independent manner due to polyclonal activation **(Bernasconi et al., 2002)**. In humans, memory B cells constitutively express specific toll-like receptors (TLRs), including TLR9. Naive B cells do not constitutively express TLR9, but it can be upregulated on naive B cells when they are stimulated through their B-cell receptors (BCRs), thus explaining the differential sensitivity of these subsets to TLR9 triggering. Given that neutralizing antibodies represent a critical line of defense, it is very likely that the immune system has evolved multiple mechanisms to maintain persistent levels of neutralizing antibody. In the present study, the Ab levels against TT vaccine remained almost stable from day 14 to day 120 after immunization. Combined with the relatively short half-life of circulating antibody and the prominent role of BM in long-term antibody production, persistent IgG serum antibody appears to be the product of BM AFCs **(Slifka et al., 1998 and Roth et al., 2014)**. Concerning The extent and role of post-GC selection in the affinity maturation of serum Ab, the affinity of serum Ab was monitored over a period of 4 mo (120 d) after primary immunization with TT vaccine and that increased numbers of BM AFCs were correlated with persistent titers of anti-tetanic IgG Ab and that increases in affinity of serum Ab for the TT antigen was maintained to the end of the experiment. It was noticed that the serum Ab avidity were decreased post

early administration of MR1 Ab compared with mice group got non MR1 Ab, these data was in alignment with **(Takahashi et al., 1998 and Han et al., 1995),** as the former directly addressed this issue by measuring the affinity of the antibody secreted by these cells in the bone marrow at various times after immunization, including times well after the histologic GC reaction had ended. These workers noted a striking increase in the average affinity of the AFCs in bone marrow at these late times. To examine the contribution to the plasma cell population in the bone marrow from the GC, they used anti-CD40L treatments to dissolve the GC reaction at various times. This approach is effective at preventing the further development of memory cells and, when used early, blocked the accumulation of high-affinity AFCs in the bone marrow, thus indicating a GC origin for these. However, when anti-CD40L was given late, after the histologic GC reaction had ended, it had no effect on the further increase in affinity of the bone marrow AFCs. This indicated that the affinity increase was not being fed by undetected GCs and instead was based on selection among the established population, in a CD40-independent way. An analysis of memory versus AFC populations in the NP response by **(Smith et al., 1997**) came to similar conclusions. Also, Administration of MR1 antibody after the end of the GC reaction had no discernible effect on TT-specific BM AFCs and consequently serum antibody levels. Thus, the progenitors of high affinity BM AFCs and GC B cells share sensitivity to suppression by the MR1 antibody during the early days of the response, and that was in agreement with **(Takahashi et al., 1998).** However, even after the GC reaction waned, affinity maturation and clonal selection continued, independent of GC structure and inhibition by MR1. Thus, the primary role for GCs in the affinity maturation of serum antibody seemed to be the generation of high affinity variants by V(D)J hypermutation; afterwards, antigen-driven interclonal selection can occur outside of the GC microenvironment in the BM. Long-term clonal selection in the post-GC environment (BM) offered the opportunity to continue to improve the antigen-selected repertoire by allowing direct competition between the progeny of both high and lower affinity GC B cells. This process did not seem to take place between the isolated B cell populations present in different GCs even when they are separated by only 20-50 μm **(Jacob et al., 1991)**. This explanation was followed by the explanation of **(Takahashi et al., 1998)** recording that despite the complete loss of GCs and splenic B cells bearing the GC phenotype, affinity maturation was present in all mice treated with MR1 antibody at days (48 and 50) after immunization. Indeed, although disruption of CD40–CD154 interactions at days (6 and 8) lowered the frequency of high affinity BM AFCs and consequently suppressed the average affinity of serum antibody, antigen-driven selection was equally efficient in MR1-treated mice at days (48 and 50) and control mice. Even the administration of MR1 antibody during the late phase (days 48–50) of the primary response did not suppress affinity maturation in the BM AFC compartment. These results demonstrated that clonal selection among BM AFCs was independent of the GC microenvironment and resistant to blockade of CD154-mediated costimulation. Selection in this population was somewhat surprising, since classically plasma cells were thought to lack surface Ig and therefore should be unable to sense the presence of Ag and be selected on that basis. It is possible that the early AFCs appearing in bone marrow are plasmablasts, rather than plasma cells **(Hauser et al., 2002).** Plasmablasts are certainly surface Ig positive and could potentially undergo affinity-based selection. It also seems likely that there are cells intermediate between surface Ig-positive, dividing plasmablasts, and surface Ig-negative non dividing plasma cells that could undergo selection, including a possibly novel "precursor" population described by Noelle and colleagues **(O'Connor et al., 2002. and Gatto et al., 2005).** This aspect of the model has yet to be fully clarified. Nonetheless, it seems clear that the early phase of AFC accumulation in the bone marrow is yet another stage at which affinity maturation takes place. In the mean time effect of MR1 administration and related effect on antibody eliciting was accepted by **(Foy et al., 1993)** recording that the average affinity (avidity) of serum Ab and BM AFCs and subsequent antibody production were not diminished by administration of the MR1 antibody after the primary GC reaction was complete. In contrast, secondary B cell responses were highly dependent on CD154-mediated costimulation as they are dramatically suppressed by MR1 antibody**.** On the contrary in the study of **Han et al., (1995)**, postulated that early administration of anti-CD40L reduced serum Ab levels to approximately 10% of controls; administration of anti-CD40L abrogated an established GC reaction. While, **Wu et al., (1995)** could explain the relation between GC diminishing and continuous elevation of antibodies level against certain Ag by using mice with targeted mutations in the genes encoding CD40L or CD28 to investigate how the CD40-CD40L interaction induces on B cells a costimulatory activity that acts in addition to antigen to trigger T-cell growth. CD40L was both necessary and sufficient for rapid, T-cell-mediated induction of costimulatory activity on B cells.

Conclusion:-

These findings suggest that GC development is the basis for high affinity antibody and crucial for optimal achievement of protective immune response and that peripheral memory B cells and long-lived BM plasma cells may represent independently regulated cell populations and may play different roles in the maintenance of protective immunity.

References:-

1. **Bemark , M. and Neuberger, M.S.(2003):** By-Products of Immunoglobulin Somatic Hypermutation. Genes, 38:32–39.
2. **Bernasconi, N. L., Traggiai, E., and Lanzavecchia, A. (2002):** Maintenance of serological memory by polyclonal activation of human memory B cells. Science 298, 2199–2202.
3. **Flajnik, MF. (2002):** Comparative analyses of immunoglobulin genes: surprises and portents. Nat Rev Immunol ., **2**(9):688–98.
4. **Flora Castellino, Grazia Galli, Giuseppe Del Giudice and Rino Rappuoli (2009)**: Generating memory with vaccination. Eur. J. Immunol. 39: 2100–210539: 2100–2105.
5. **Foy, T.M.; Shepherd, D.M.; Durie, F.H.; Aruffo, A.; Ledbetter, J.A. and Noelle, R.J. (1993):** In vivo CD40–gp39 interactions are essential for thymus dependent humoral immunity. II. Prolonged suppression of the humoral immune response by an antibody to the ligand for CD40, gp39. J. Exp. Med.178:1567–1575.
6. **Gatto, D.; Pfister, T.; Jegerlehner, A.; Martin, S.W.; Kopf, M. and Bachmann M.F.(2005):** Complement receptors regulate differentiation of bone marrow plasma cell precursors expressing transcription factors Blimp-1 and XBP-1 JEM ,21(6): 993–1005.
7. **Gatto, D. and Brink, R.(2010):**The germinal centre reaction**.**J. Allerrgy Clin Immunol., 126: 901-910.
8. **Han, S.; Hathcock, K.; Zheng, B.; Kepler, T.B.; Hodes, R. and G. Kelsoe G. (1995):** Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. J. Immunol. 155:556–567.
9. **Hassel, B. (2013):** Tetanus: Pathophysiology, Treatment, and the Possibility of Using Botulinum Toxin against Tetanus-Induced Rigidity and Spasms. Toxins, 5, 73-83.
10. **Hauser, A. E., Debes, G. F., Arce, S., Cassese, G., Hamann, A., Radbruch, A., and Manz, R. A. (2002).** Chemotactic responsiveness toward ligands for CXCR3 and CXCR4 is regulated on plasmablasts during the time course of a memory immune response. J Immunol 169, 1277–1282.
11. **Jacob, J.; Kassir, R. and Kelsoe, G. (1991):** In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl) acetyl. I. The architecture and dynamics of responding cell populations. J. Exp. Med. 173:1165–1175.
12. **Jacob, J.; Przylepa , J. Miller , C. and Kelsoe, G.(1993):** In – situ studies of the primary immune response to (4-hydroxyl -3- nitrophenyl) acetyl. The kinetics of V region mutation and selection in germinal center B cells. J. Exp. Med . 178: 1293-1307.
13. **Manz, R.A., Thiel, A. and Radbruch, A. (1997):** Lifetime of plasma cells in the bone marrow. Nature. 388:133–134.
14. **Mouquet, H.; Scheid, J.F.; Zoller, M.J. et al. (2010):** Polyreactivity increases the apparent affinity of anti-HIV antibodies by heteroligation, Nature 467 (2010) 591–595**.**
15. **O'Connor, B.P.; Cascalho, M. and. Noelle, R.J. (2002):** Short-lived and Long-lived Bone Marrow Plasma Cells Are Derived from a Novel Precursor Population J. Exp. Med. 195, (6), 737–745.
16. **Pullen, G.R.; Fitzgerald, M.G. and Hosking, C.S. (1986):** Antibody avidity determination by ELISA using thiocyanate elution, J. Immunol. Methods 86: 83–87.
17. **Romero-Steiner, S.; Musher, D.M.; Cetron, M.S.; et al. (1999):** Reduction in functional antibody activity against Streptococcus pneumoniae in vaccinated elderly individuals highly correlates with decreased IgG antibody avidity. Clin Infect Dis 29:281–288.
18. **Roth, K.; Oehme, L.; Zehentmeier, S.; Zhang, Y.; Niesner, R.; Hauser, A.E. (2014):** Tracking Plasma Cell Differentiation and Survival Cytometry Part A, 85A: 15-24.
19. **Slifka, M.K.; Antia, R.; Whitmire, J.K. and Ahmed ,R. (1998):** Humoral Immunity Due to Long-Lived Plasma Cells Immunity, 8, 363–372.
20. **Smith, K.G.C.; Light, A.; Nossal, G.J.V. and Tarlinton, D.M. (1997):** The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response. EMBO (Eur. Mol. Biol. Organ.) J. 16: 2996–3006**.**
21. **Takahashi, B.Y.; Dutta, P.R.; Cerasoli, D.M. and Kelsoe, G. (1998):** In Situ Studies of the Primary Immune Response to (4-Hydroxy-3-Nitrophenyl) Acetyl. V. Affinity Maturation Develops in Two Stages of Clonal Selection. J. Exp. Med. 187(6): 885–895.
22. **Tarlinton, D.M. (2008):** Evolution in miniature: selection, survival and distribution of antigen reactive cells in the germinal centre. Immunol. Cell Biol. 86, 133–138.
23. **Van Riet, E.; Retra, K.; Adegnika, A. A.; Jol-van der Zijde, C. M. ; Uh, H. W.; Lell, B.; Issifou, S. ; Kremsner, P. G.; Yazdanbakhsh, M. Van Tol, M. J. and Hartgers, F. C.(2008):** Cellular and humoral responses to tetanus vaccination in Gabonese children. Vaccine **26:**3690–3695.
24. **Wu, Y.; Xu, J.; Shinde, S.; Grewal, I.; Henderson, T.; Flavell, R.A. and Liu, Y.(1995):** Rapid induction of a novel costimulatory activity on B cells by CD40 ligand. Curr Biol. , 1;5(11):1303-11**.**
25. **Zhang, W.; Bardwell, P.D.; Woo, C.J.; Poltoratsky, V.; Scharrf, M.W. and Martin, A. (2001):** Clonal instability of V region hypermutation in the Ramos Burkitts lymphoma cell line. International Immunology, 13(9): 1175-1184.