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Generation of T cell hybridoma as a technique for study the immune response against bacterial infections

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ABSTRACT

Study of the acquired immune responses against microbial infection has a high importance, as it is the fundamental basis of designing the vaccines against microbes and production of specific antibodies against infections. Different methods have been established to study the immune responses against infections using different types of immune cells. The main cells of acquired immune system are T cells which generate cellular immune response and B cells that produce humoral immune response. One of the cellular techniques can be generated, as a continues cell system is called a hybridoma. This cell system is used to study the immune responses of T and B cells of the immune system. It can be generated from T cell lines to study cellular immunity, and is called T cell hybridoma, or using B cell lines to study humoral immunity, and is called B cell hybridoma. Generation of T cell hybridoma (a fusion between antigen-specific primary T cells with an immortal thymoma line) is a significant technique to demonstrate the mechanisms of antigen presenting to T cells. In addition, it is a basic technique for production of monoclonal antibody based on the fusion of B cell lines (i.e., B cell hybridoma). This article aims to present the procedure of generating T cell hybridoma and its application to study the cellular immune response against M5 protein of Streptococcus pyogenes (group A Streptococcus - GAS) as a practical example. It is an important issue to highlight the methods of such successful technique.

تخليق الخلايا الهجينة للخلايا التائية كتقنية لدراسة الإستجابة المناعية ضد الإصابات البكتيرية

جميلة على حامد الحضيري

قسم على الحيوان، كلية العلوم، جامعة سبها ، ليبيا

الكلمات المفتاحية:
حليل الأنتيجين
عرض الأنتيجين
خلايا عرض الأنتيجين
الخلايا الهجينة التائية
الخلايا الهجينة البائية
الاستجابات المناعية

الملخص

تعتبر دراسة الاستجابة المناعية المكتسبة ضد الاصابات الجرثومية ذات أهمية كبيرة حيث تمثل القاعدة الأساسية لتصنيع اللقاحات ضد الميكروبات وانتاج الاجسام المضادة المتخصصة ضد هذه الاصابات. يمكن استحدام طرق عديدة ومختلفة لدراسة الاستجابة المناعية ضد الاصابات وباستعمال أنواع مختلفة من الخلايا المناعية. والخلايا الرئيسية للجهاز المناعي المكتسب هى الخلايا التائية والتى تكون الاستجابة المناعية الخلوية عن طريق انتاج خلايا مناعية فعالة ، و كذلك الخلايا البائية التى ينتج عنها الاستجابة المناعية الخلطية وذلك عن طريق انتاج الاجسام المضادة المتخصصة. احدى التقنيات الخلوية المستخدمة فى دراسة الاستجابة المناعية الخلوية والخلطية ، ويمكن أن تصنع فى معمل زراعة الأنسجة كنظام خلوي مستمر اى دائم النمو هو تخليق ما يعرف بالخلايا الهجينة ويمكن أن تصنع من الخط الخلوي للخلايا التائية وذلك لدراسة الاستجابة المناعية الخلوية وتسمى الخلايا الهجينة التائية، أو يمكن تصنيعها من الخط الخلوي البائي للخلايا البائية لدراسة الاستجابة المناعية الخلطية ويطلق عليها الخلايا الهجينة البائية. يتم تخليق الخلايا الهجينة التائية بخلط الحط الخلوى الطبيعي للخلايا التائية المتخصصة للأنتيجين المراد دراسة الية عرضه للخلايا التائية مع الخط الخلوي السرطانى للخلايا التائية . فى حين أن الخلايا الهجينة البائية يتم دمج الخط الخلوى الطبيعى مع الخط الخلوي السرطانى من الخلايا البائية وتستخدم لانتاج الاجسام المضادة وحيدة النسخة والمتخصصة لأنتيجين معين. تهدف هذه الورقة الى عرض طريقة تخليق الخلايا الهجينة التائية المتخصصة لأحد البروتينات

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المكونة لبكتيريا ستربتوكوكس النوع الأول كمثال تطبيقي، واستعمالها فى دراسة الاستجابة المناعية الخلوية ضده. ومن الأهمية أن يتم عرض هذه التقنية لما حققت من نجاح فى الدراسات السابقة.

Introduction

B and T lymphocytes are the antigen-specific cells of the immune system which require antigen (Ag) stimulation to progress. Inasmuch, specific responses to pathogens or foreign substances demand recognition of the antigen and induce a signal transduction by the receptors on the surface of these cells leading to the initiation of adaptive immune responses **[1]**. Their fundamental function is largely mediated by secretion of a set of small proteins (known as cytokines). These cytokines act by binding to high-affinity receptors expressed on the target cells and by inducing biochemical signals within these cells **[2]**. As a result, the cytokines activate phagocytic cells to internalize pathogenic organisms and eliminate them. In addition, the induction of T cells stimulates B cells to produce antibodies (Ab) specific to the antigen **[2]**.

Once T cells are activated, they recognise small regions (epitopes) of the antigenic proteins bound to the major histocompatibility complex (MHC), expressed on the surface of other cells known as antigen presenting cells (APC). The epitopes are generated by modification of the native protein usually followed by their enzymatic degradation inside APC, referred to as 'antigen processing'. The resulting peptides bind MHC molecules which are transported to the APC surface for presentation to T cells. The latter process is known as 'antigen presentation' **[3]**. This mechanism is related to acquired immune response where CD4 T cells (helper T cells) and CD8 T cells (Cytotoxic T cells) are the responsible cells. In case of CD4 T cells immune response, MHC class-II molecules are loaded with peptide fragments during biosynthetic assembly of the molecules to present the binding peptides to $CD4^+$ T cells. Whereas, for CD8 T cells immune response, MHC class-I molecules are enrolled in the mechanism of antigen processing and presentation **[4]**.

Study of the acquired immune responses against microbial infection is at a high importance, as it is the fundamental basis of designing the vaccines against microbes and production of specific antibodies against infections. Several studies have been carried out in this aspect, for example, studies on *Bacillus anthracis*, which is an endemic infection worldwide, focussing on determination of CD4 T cell immune responses **[5-8]**. Other research aiming at better understanding of adaptive immunity to *Burkholderia pseudomallei*, which is the bacterial pathogen of melioidosis **[9-10]**. Another study looked at the mechanisms of processing and presentation of *Yersinia pestis*' V antigen **[11]**. Also, Musson et al., studied the processing of viable *Salmonella typhimurium* from its invasion protein **[12]**.

Different methods have been used to study the immune responses against infections using different types of immune cells. The main cells of acquired immune system are T cells which generate cellular immune response and B cells that produce humoral immune response. One of the cellular techniques that can be generated, as a continues cell system is called a hybridoma. This cell system is used to study the immune responses against foreign antigens. It is called T cell hybridoma to study cellular immunity if using T cell lines, and it is called B cell hybridoma in case of using B cell lines to study humoral immunity, **[13]**. B cells hybridoma was developed before the generation of T cells hybridoma **[14]**. Subsequently, T cell hybridomas were generated by other earlier studies **[15-16]**.

Generation of T cell hybridoma (a fusion between antigen-specific primary T cells with an immortal thymoma line) is a significant technique to demonstrate the mechanisms of antigen presenting to T cells. In addition, it is a basic technique for production of monoclonal antibody based on the fusion of B cell lines (i.e., B cell hybridoma).T cell hybridomas have more advantages than primary T cell clones, in terms of their uniformity, stability long time, and their availability in large numbers in the lab for antigen presentation experiments **[13]**.

Previously, it was used T cell clones specific for an antigen to study immune responses , such as: T cell clones specific to $OVA_{323-339}$ **[17]**, presentation of an epitope P_{18-29} in the matrix protein of influenza virus and $P_{307-318}$ epitope in the hemaglutinin (HA) protein of the same virus **[18]** , and presentation of I-E^d-restricted epitope of hemagglutinin protein of influenza virus ($HA_{302-313}$) and $HA_{107-119}$ epitopes to the immune system using the specific cell lines for both epitopes **[19]**. Testing of epitopes from a parasite (*Plasmodium chabaudi chabaudi*) showed their induction to stimulate T cell responses using T cell clones specific for these epitopes **[20]**. A series of studies have been carried out to demonstrate the mechanisms of antigen processing and presentation of the bacterial antigens. Previous studies have mapped T cell epitopes on M5 protein of *Streptococcus pyogenes* which were recognized by many T cell clones specific for each one and different mouse strains **[21-22]** .

Moving to T cell hybridomas specific for an antigen instead of T cell clones, several previous studies have generated T cell hybridomas and applied them for study the presentation of different antigens to the immune system. The results of such studies **[5-8, 11,12]** have demonstrated an effective processing of bacterial antigens and binding of antigenic fragments to MHC class II molecule for presentation to CD4 T cells. Two epitopes from streptococcal M5 protein ($M5_{17–31}$ and $M5_{308–319}$) have been studied to investigate the mechanism of Ag processing and presentation using mouse macrophages and T cell hybridomas specific for each epitopes **[23-29]**. This approach is the basis for bacterial vaccines development. T cell hybridomas was generated and applied for study of processing of viruses as well, such as HIV and HCV **[30-33]**. In terms of non-bacterial disease, T-cell hybridomas specific for aggrecan which is a structural glycoprotein of cartilage and candidate autoantigen in rheumatoid arthritis were also generated. These hybridomas recognized epitopes from domain of aggrecan **[34-36]**. This article aims to present the procedure of generating T cell hybridoma and its application to study the cellular immune response against M protein of *Streptococcus pyogenes* (group A Streptococcus - GAS) as an example. It is an important issue to highlight the methods of such successful technique.

2. Materials and methods

2.1 Antigens

Synthetic peptides covering two T cell epitopes on the M5 protein of *S. pyogenes*: $M5_{1-35}$ peptide (AVTRGTINDPQRAKLDKYELENHDLKTKNEGLK) was synthesized by the Molecular Biology Unit, Newcastle University , UK and $M5_{359-388}$ peptide (LAKQAEELAKLRAGKASDSQTPDTKPGNKA) was purchased from GenScript Corp.USA. The Manfredo strain of type 5 *Streptococcus pyogenes* was obtained from M. A. Kehoe, Department of Microbiology, Newcastle University.

2.2 Mouse strain and immunization

C57BL/6 strain mice ($H\text{-}2^b$ haplotype) were purchased from Bantin and Kingman, Hull, GB. They were used for immunization with peptides $M5_{1-35}$ and $M5_{359-388}$ of streptococcal M5 protein (at 6mg/ml of each) . All mice were footpad immunized with 150 µg/ml per mouse of each peptide (1:1 in Titremax adjuvant). Four mice /experiment were used for immunization (2 mice for each peptide).The experiment was repeated twice.

2.3 Culture media

The culture media were purchased from Sigma company . The following media were used in the current study: RF10 medium consists of RPMI 1640 medium containing 3 mM L-glutamine, 50 µM 2-mercaptoethanol, 10% foetal bovine serum (FBS), and 30 µg/ml gentamycin. Bone marrow (BM) medium was used for growing bone marrow macrophages **(**BMM). It contains RPMI 1640 medium , 3 mM L-glutamine, 50 µM 2-mercaptoethanol, 10% fetal bovine serum (FBS) , 5% horse serum , 1mM sodium pyruvate, 10 mM herpes buffer and 30 µg/ml gentamycin. It is also supplemented with 5% of a supernatant from the L929 cell line as a source of macrophage colony stimulating factor (M-CSF). Microbiological culture medium was used for growing the bacteria (the Manfredo strain of type 5 *S. pyogenes*). It is prepared by mixing 3% of Todd-Hewitt broth containing 1% Yeast Extract (Difco Laboratories, Detroit, MI), and sterilised by autoclaving at 121º C for 15 min.

2.4 Tissue culture

2.4.1 $M5_{1-35}$ - specific T cell line

T cells were grown by culture of popliteal lymph node cells from ($M5_{1-35}$) – footpad immunized C57BL/6 mice in 24-well plates ($5*10^6$ cells / well).They were stimulated by the peptide and incubated for 72 hours at 37 °C in a humidified CO_2 incubator. Ficolling on the third day of culture and interleukin-2 (IL-2) was added for T cells growth. The medium was changed every 3-4 days and the cells were restimulated with γ-irradiated spleen cells from C57BL/6 strain mice and Ag every 14 days for 3-4 times before using the line. T cell line specific for heat-killed *Streptococcus pyogenes* (HK-S) was developed by restimulation of two wells of ($M5_{1-35}$) - specific line with HK-S at $3*10^{8}$ CFU /ml (10^7 CFU /ml). The line was restimulated with viable Streptococci at $3*10^8$ CFU /ml ($3*10^6$ CFU /ml). The same procedure was done to generate $M5_{359-388}$ - specific T cell line.

2.4.2 Culture of bone marrow macrophages (BMM)

Bone marrow-derived macrophages were grown from femoral bone marrow cells of C57BL/6 strain mice to use as APC. They were seeded in Petri dishes maintaining in BM medium for a week at 37 °C in a humidified 5 % CO_2 incubator. Before using macrophages in the assay, they were activated by treatment with 1 ng/ml interferon-γ (R&D Systems, Abingdon, UK) and incubated overnight at 37 °C.

2.5 T cell proliferation assays

2.5.1 Mapping the epitopes

This assay aims to map epitopes on the immunized peptides by measuring T cell response against the primed antigen. It was performed by the culture of popliteal lymph node cells (PLNs) - T cells from ($M5_{1-35}$) and ($M5_{359-388}$) – footpad immunized C57BL/6 mice (after 7 – 10 days of immunization) into a round bottomed 96 – well plate ($3*10^5$ cells / well). The antigen was added as triplicate wells of 3 doses at 0.8, 4 and 20µg/ml. The cultured plate was incubated for 72 hours at 37 °C in a humidified 5 % CO_2 incubator. Staphylococcus aureus enterotoxin (SEB) at $3*10^{-7}$ M was used as a positive control . The cultured cells were pulsed-labelled for the last 24 hours of incubation with 14.8 KBq of ^{3}H-T (TRA310, specific activity 74 GBq/mmol , Amersham Bio-sciences.UK). Further incubation occurred for 18-20 hours. Harvesting was done onto glass fibre filters on the following day of pulsing using a cell harvester (Inotech . Switzerland). It was left to dry for 24 hours. The radioactivity was counted using a direct β – counter (Matrix 9600, Packard Instrument Company, Meridan, CT). The results are represented as mean counts per minute (CPM) of each triplicate wells ± S.D . If the mean CPM was two-fold or more than negative control the T cell response was positive.

2.5.2 Specificity of $M5_{1-35}$ - specific T cell line

T cell proliferation assay was also done to test the ($M5_{1-35}$) -T cell line for its specificity to the peptide, HK-S and viable streptococcus as a source of M5 protein**.** It was carried out by culturing T cells ($5*10^4$/well) and γ-irradiated spleen cells ($75*10^4$/well) from C57BL/6 strain mice into a round bottomed 96 – well plate. Three doses of ($M5_{1-35}$) peptide at 2mg/ml (0.8, 4 and 20µg/ml), 3 doses of HK-S and viable streptococcus at $3*10^8$ CFU /ml (10^6, $3*10^6$ and 10^7 CFU /ml) were added to the plate as triplicate wells. Concanavalin A (Con-A) at 1mg/ml was used as a positive control. The same procedure was done to test the specificity of $M5_{359-388}$ - specific T cell line.

2.6 $M5_{1-35}$ - specific T cell hybridomas

T-cell hybridomas specific for $M5_{1-35}$ were generated by polyethylene-glycol fusion of BW5147 ($TCR^{\alpha-\beta-}$) cells (provided by Dr. P. Marrack, Departments of Microbiology and Immunology, and Medicine, University of Colorado Health Sciences Centre, Denver) with $CD4^+$ T-cell line from ($M5_{1-35}$) -immunized C57BL/6 strain mice ($H\text{-}2^b$ haplotype). It was applied as the procedure established at immunology lab at Newcastle University- School of Clinical Medical Sciences. The same procedure was done to generate $M5_{359-388}$ - specific T cell hybridoma.

2.7 Specificity of T-cell hybridomas

Hybridomas were screened to determine their specificity to antigen; $M5_{1-35}$ and *Streptococcus pyogenes*, using spleen cells or BMM ($4*10^4$ /well) as APC from C57BL/6 mice. The spleen cells or BMM were cultured in a flat-bottomed 96-well microtiter plate with antigen in triplicate wells and $M5_{1-35}$ T cell hybridomas ($4*10^4$ /well) . Con-A at 1mg/ml was used as a positive control. The cultured cells were incubated for 20-24 hours, and put in -30 freezer for a further 24 hours. Before the assay, CTLL-2 cells were washed twice and cultured without IL-2 overnight. CTLL assays were done by culturing CTLL-2 cells (4×10^4/well) in triplicate wells of flat-bottomed 96-well microtiter plate mixed with supernatants of T cell hybridoma culture. Interleukin-2 (IL-2) was used as a positive control. The cells were incubated for 6-12 hours, then pulse-labelled with 14.8 KBq of ^{3}H-T. A Further incubation took place for 18-20 hours before harvesting onto glass fibre membranes. A direct beta counter was used to measure the response as mean CPM of triplicate wells ± S.D. The response of T-cell hybridomas in these assays was measured as the amount of interleukin-2 released as proliferation of CTLL-2 cells. The background (non IL-2) should be less than 100 CPM . Positive results were considered if the mean CPM was two-fold or more than the background. A CTLL control assay must be done for each experiment. The same procedure was done to test the specificity of $M5_{359-388}$ - specific T cell hybridoma.

2.8 Antigen uptake

Macrophages were fixed in two steps ; pre-fixation (before adding antigen) to demonstrate antigen uptake and determine whether the antigen requires processing or not (this indicates the structure of the protein), and post-fixation (after antigen has been added) to study the kinetics of antigen processing. Un-fixed antigen-pulsed macrophages were used as a control. The cells were incubated for 5 hours after adding antigen then fixed in 1% paraformaldehyde for 5 minutes to

block Ag uptake by BMM. Fixation was stopped with 0.05% Gly-Gly and the cells washed 3 times with HANKS (balanced salt solution).Then T-cell hybridomas (4×10^4/well) were added to the antigen-pulsed macrophages, and plates were incubated for 24 hours. The viability of the cells was confirmed by light microscopy before putting them in -30 freezer. After 24 hours of freezing, culture supernatants were thawed and used in CTLL assays.

2.9 Antigen presentation assays using $M5_{1-35}$ - specific of T cell hybridomas

These assays were carried out as indicated priory using spleen cells or BMM as APC. These assays were done once to test the specificity of $M5_{1-35}$ - specific T - cell hybridomas. T cell hybridomas (TCHs) specific for $M5_{1-35}$ were assayed for their specificity to $M5_{1-35}$ peptide and to HK-S (the Manfredo strain of type 5 *S. pyogenes*) as a source of M5 protein by using spleen cells or BMM from C57BL/6 ($H\text{-}2^b$) as APC . The peptide was at 2mg/ml an three doses of it were used (0.8, 4 and 20µg/ml or 5, 10 and 20µg/ml). The concentration of HK-S was adjusted by ELISA reader (TECAN microplate reader) at $3*10^8$ CFU /ml (A_{600} = 0.6) and three doses were used (10^7, $3*10^7$ and 10^8 CFU /well). ConA (1mg/ml) was used as a positive control in these assays. The same procedure was done for antigen presentation assay of $M5_{359-388}$ - specific T cell hybridoma.

2.10 Statistical analysis

Microsoft Prism 4.0 was used for descriptive statistics to calculate the mean , standard error of the mean (SEM) and the standard deviation (S.D) of the data as triplicate wells.

3. Results

3.1 T cell proliferation assays

3.1.1 T cell responses

Two C57BL/6 mice ($H\text{-}2^b$ haplotype) were immunuzed with $M5_{1-35}$ and two with $M5_{359-388}$ peptides of streptococcal M5 protein to identify T-cell epitopes recognised by T cells from C57BL/6 mice by measuring T cell response to each peptide. PLN from ($M5_{1-35}$) and ($M5_{359-388}$) - immunized C57BL/6 mice were assayed for their responsiveness to these synthetic peptides. T-cell proliferation response to the immunizing peptide at the doses used was clearly observed (Figure. 1).

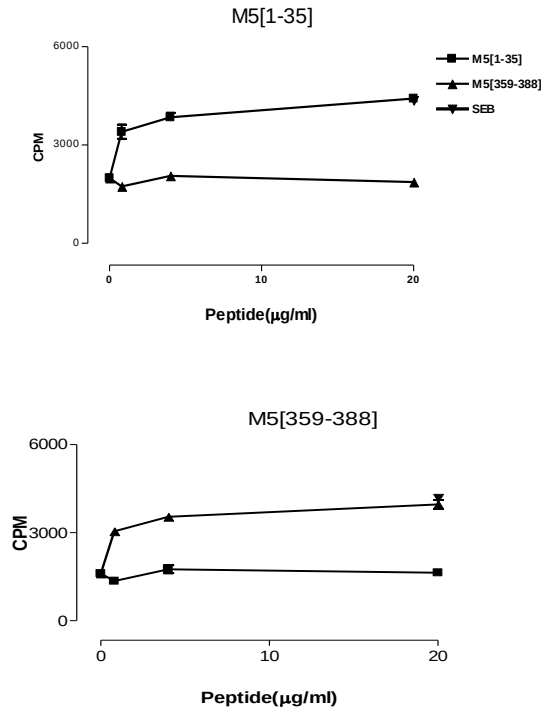

Figure. 1 T cell proliferation from PLN- immunized mice. Two C57BL/6 mice were immunized with $M5_{1-35}$; **(upper)** and $M5_{359-388}$; **(lower)** of streptococcal M5 protein to map T-cell epitopes on each peptide. Three doses of peptide (0.8, 4 and 20 µg/ml) were used. T-cell response to the immunized peptide at the used doses was clearly observed. SEB was use as a positive control. The result was plotted as mean CPM of triplicate wells ± S.D.

3.1.2 Specificity of $M5_{1-35}$ - specific of T cell line

T cell line for $M5_{1-35}$ was tested for its specificity to the peptide, HK-S and viable streptococcus as a source of M5 protein**.** T cells cultured as mentioned in the methods and a proliferation assay was carried out. Firstly, it was used BMM pre-treated with IFN-γ as APC for the assay, but the T cells did not recognize both HK-S and the peptide. Then, γ-irradiated spleen cells were used. The data showed the responsiveness of T cell line to the peptide , less response to HK-S at lower dose (10^6 CFU /ml) , but there was no response to viable streptococci (Figure.2).

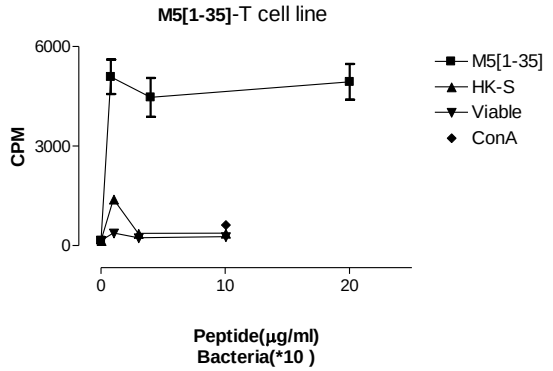

Figure.2 Specificity of T cell line for $M5_{1-35}$. T cell line for $M5_{1-35}$ was tested for its specificity to the peptide , heat–killed streptococcal (HK-S) and viable streptococci. The γ-irradiated spleen cells were used as APC. Three doses (0.8, 4 and 20µg/ml) of $M5_{1-35}$ peptide at 2mg/ml and 3 doses of HK-S and viable streptococci at $3*10^8$ CFU /ml (10^6, $3*10^6$ and 10^7 CFU /ml) were used. The result showed a positive response of T cell line to the peptide, less response to HK-S at lower dose (10^6 CFU /ml) , but no response to viable streptococci. Concanavalin A (Con-A) at 1mg/ml was used as a positive control. T cells proliferation was measured as mean CPM of triplicate wells ± S.D.

3.2 $M5_{1-35}$ - specific of T cell line and hybridomas

T cell line from PLN-immunized mice (H-2^b) with $M5_{1-35}$ of streptococcal M5 protein - specific for $M5_{1-35}$ was successful and T cell hybridomas specific for this peptide were generated. Growing T cell line and generating T cell hybridomas take a long time to complete (about 2 months for each).T cell hybridomas specific for $M5_{1-35}$ peptide were generated from a fusion between T cell line specific for $M5_{1-35}$ with BW5147 ($TCR^{-}\alpha^{-}\beta^{-}$) cells. Twelve T cell hybridomas (TCH_1 - TCH_{12}) were obtained and Ag presentation assays were carried out.

3.3 Ag presentation assay using $M5_{1-35}$ - specific T cell hybridomas

3.3.1 Specificity of T-cell hybridomas

T cell hybridomas (TCHs) specific for $M5_{1-35}$ of streptococcal M5 protein were assayed for their specificity to $M5_{1-35}$ peptide and to heat-killed streptococci (HK-S) as a source of M5 protein by using spleen cells from C57BL/6 (H-2^b) as APC . A significant responses to the peptide was shown by TCH-1, TCH-3, TCH-5 and TCH-9 (Figure.3).There was a much lower or no response either to the peptide or to the HK-S by the other hybridomas (data not shown). On the other hand, all the TCHs failed to recognize HK-S. However, there was a lower response when using the lower dose of HK-S (10^7 CFU /well) in contrast with the higher doses ($3*10^7$ and 10^8 CFU /well).CTLL control assays were done for each experiment as shown previously.

Therefore, the results have shown that some T-cell hybridomas have specificities to the peptide, but all failed to recognize HK-S. The responded hybridomas were tested for their responsiveness to the antigen using BMM as APC. All these hybrids showed responses $M5_{1-35}$ peptide except TCH-3. In addition, TCH-12 was tested for its specificity using BMM and viable bacteria instead of HK-S. A high immune response to the peptide was clearly seen but there was no response to the viable bacteria (Figure.4). Thus, the viable bacteria also could not recognized by the TCH. I suggest applying some modification on the viable bacteria to facilitate Ag processing.

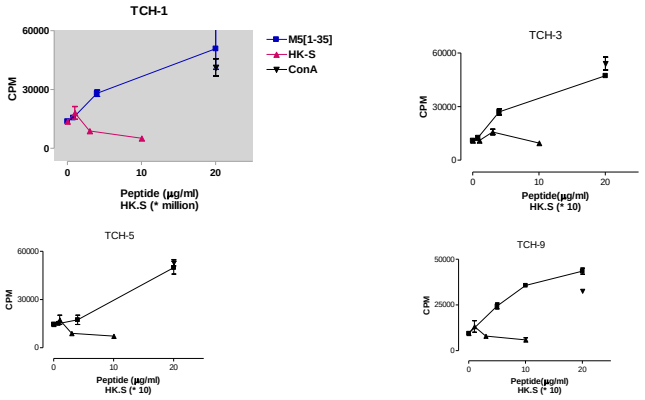

Figure.3 Presentation of $M5_{1-35}$ peptide of streptococcal M5 protein and heat-killed streptococci (HK-S) by spleen cells. T cell hybridomas (TCHs) specific for $M5_{1-35}$ peptide of M5 protein were assayed for their specificity to $M5_{1-35}$ peptide and to heat-killed streptococci (HK-S) as a source of M5 protein using spleen cells ($4*10^4$/well) as APC. Three doses of peptide (0.8, 4, and 20 µg/ml) or (5, 10 and 20 µg/ml) were used in these assays . Four T cell hybridomas (TCH-1; **upper left,** TCH-3; **upper right**, TCH-5; **lower left** and TCH-9; **lower right**) presented significantly the peptide. ConA (1mg/ml) was used as a positive control in these assays. The responses of T-cell hybridomas were measured as CTLL proliferation as mean CPM ± S.D.

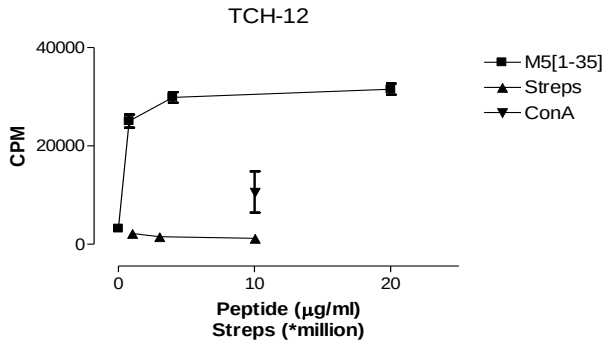

Figure.4 Ag presentation assay using BMM and TCH specific for $M5_{1-35}$ peptide. TCH-12 T cell hybridoma specific for $M5_{1-35}$ peptide of streptococcal M5 protein was tested for its specificity to the peptide and to the viable streptococci (streps) as a source of M5 protein using BMM from C57BL/6 mice at ($4*10^4$/well) as APC.

Three doses of peptide (0.8, 4, and 20 µg/ml) at 2mg/ml and the viable streptococci (10^6, $3*10^6$ and 10^7 CFU /well) at $3*10^8$ CFU /well were used in this assay . The responses of T cell hybridomas were measured as CTLL proliferation as mean CPM ± S.D.

4. Discussion

The methods applied in this study were established by immunology lab at Newcastle University and have been applied in several studies and showed successful results **[5-12]**. The results of the example study presented in this article have indicated that the generated T cell lines and T cell hybridomas specific for M5 of Streptococcus pyogenes did recognise both immunogenic peptide from this protein and did not recognize HK-s and viable Streptococcus pyogenes. That explains the necessity of immunization of mice with the whole M5 protein of the bacteria, so we can study the processing of the antigen **[11-12]**. This work highlights the useful application of T cell hybridoma for study immune responses against microbial antigens. In terms of non-bacterial disease, T-cell hybridomas specific for aggrecan were also successful tool to study the mechanisms of immune responses to arthritis **[34-36]**. On the other hand, the identification of an antibody from a hybridoma to T cell expression has been shown as a straightforward protocol for treatment of B cell cancer **[37]**. It has been concluded that generation of T cell hybridoma has advantages in terms of their uniformity, stability long time, and their fast growing in large numbers and that facilitates carrying out extensive experiments of antigen presentation assays **[13]**. Phenotyping of the generated T cell hybridoma is important to demonstrate the expression of T cell receptors on the surface of the hybridoma. In conclusion, this cellular system technique is a useful tool in tissue culture laboratory.

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Ab: Antibody
Ag: Antigen
APC: Antigen presenting cell
BMM: Bone marrow macrophages
CFU: Colony forming unit
Con-A: Concanavalin A
CPM: Counts per minute
CTLL-2: Cytotoxic T cells
DC: Dendritic cells
GAS: Group A Streptococcus
HK-S: Heat-killed Streptococcus
^{3}H-T: Tritiated thymidine
IFNγ: Interferon gamma
IL-2: Interleukin-2
MHC: Major histocompatibility complex
PLN: Popliteal lymph node
SEB: Staphylococcus aureus enterotoxin
TCH: T cell hybridomas
TCR: T cell receptor