Tests detecting cellular immune responses

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Cell mediated immune response (CMI)

CMI is especially important for destroying intracellular bacteria and eliminating viral infections.

Classical approach:
- Antibody mediated immunoresponse (B-cells)
- Cell mediated immunoresponse (T-cells)

Diagnostic tests based on CMI (macrophages and T-cells activity)
Traditional assays analyzed bulk populations of T cells for proliferation (by \( ^3\text{H}-\text{thymidine incorporation} \)) or for cytotoxicity (by 51Cr release assays). These methods tend to be long and labor-intensive, and their results usually cannot be compared quantitatively.

“Hightech assays” of antigen-specific T cells have come into use:
- MHC-peptide tetramer staining,
- enzyme-linked immunospot (ELISPOT) assays,
- intracellular cytokine assays,
- gene expression...

CMI diagnostic techniques:
- Blastogenesis
- In vitro cytokines detection (IFN-\( \gamma \), TNF-\( \alpha \), IL…)
- Differential gene expression
- In vivo Delayed Type Hypersensitivity (DTH) reaction
Lymphocite Blastogénesis Tests

When T cells meet their specific antigen they are stimulated to undergo division. This mitogenic response is usually accompanied by morphological change to a blast cell.

The degree of lymphocite stimulation can be assayed either by determining the number of blast cells in the culture or by quantifying the amount of radioactive DNA incorporated into newly synthesized DNA.

Sample: heparinized blood (peripheral blood mononuclear cells extract)

Method:

Lymphocites extraction

Lymphocites are accounted and cell concentration is adjusted.

Account of T-cells:
- Scintillator ($^{3}$H-thymidine)
- Colorimetric reaction
- Flow citometry

Incubation

Cells are cultured in tubes or microplates with adequate tissue medium.

Optimum concentration of antigen is added for lymphocites stimulation.

Negative control: cells cultured without antigen

Mitogenic control: non-specific mitogen
In vitro cytokines detection after T-cell stimulation

T-cells from infected animals, when exposed and incubated with the specific antigen *, produce cytokines...

* The antigen must be compound by proteins / peptides to elicit specific CMI

**Cytokines**

- IFN-γ. Commonly used for diagnostic purposes
- Interleukines (IL2, IL4...)

**Samples**

- Blood (complet heparinized blood or peripheral blood mononuclear cells)
- Lymphoid tissues, Spleen...

**Methods**

Cytokines production can be quantified by different methods:

- ELISA
- ELISPOT / FLUOROSPOT
- FLOW CITOMETER / LUMINEX
- INTRACELLULAR CYTOKINE STAINING
ELISA (sandwich enzyme-linked immunosorbent assay)

+ substrate

Colour reaction quantified by spectofotometry

Antibody anti IFN-γ

IFN-γ secreted after stimulation of the sample with the specific antigen

Antibody anti IFN-γ conjugated (HRP, Streptavidin...)
In ELISPOT assays, cellular secretions are captured around the originating cell and manifested as colored secretory footprints. This allows the frequencies of the secreting cells to be measured with an extraordinary level of precision and accuracy. Basically, each cell leaves a “footprint.”
Flow cytometry

Laser

Dichroic mirrors

Nozzle

Detectors

Filters

Electronic processing

Sample stream with cells

Stream separates into droplets containing cells that can be sorted

Detector pulse

Peak height

Area

Time

Dot plot

1 cell = 1 dot

Green fluorescence intensity

Red fluorescence intensity
**Intacellular cytokines staining**

The samples are stimulated with the specific antigen in the presence of a secretion inhibitor (BFA), allowing for intracellular accumulation of newly synthesized cytokines.

**Lysis of erythrocytes and fixation of leucocytes**

Surface and intracellular staining antibodies are added in a single staining step.

**The cells are washed and fixed for flow cytometric analysis**

![Diagram](image.png)

**Figure 1** Schematic of whole blood FastImmune antigens-specific assay: Part A. From blood draw to sample activation to flow cytometric sample processing. Part B: Staining and processing of samples for flow cytometric analysis, applies to tubes 1 to 4 from Part A.
Differential gene expression

- Macrophages or T-cells are activated with the specific antigen
  Incubation → The IFN-γ or T-cells mRNA expression is determined by microarray hybridization or real-time PCR

- Characterization of the differential expression of inflammatory and immune response genes (look for up-regulated or down-regulated genes in infected animals vs uninfected ones)

**Microarray hybridization**
In the animal health context...

Most of these tests are used exclusively for research purposes:
- Monitoring of immune status during disease
- Vaccine efficacy studies

- Expensive equipment and reagents are required
- The setting up is quite labourious. Optimal reagents (adequate antigen, reagents, controls, sample dilution, reagent concentrations...) and conditions (temperatures, incubation times...).
- Highly experienced staff is required to perform the assay and interpret results correctly

Standarization and validation for the obtention of comparative results is almost impossible (results can be highly variable from one lab to another).
Just the INF-γ assay (assessed by ELISA) is applied for diagnostic purposes in control and eradication programs (e.g., Tuberculosis).

**Advantages**

- Useful when cellular immune response predominates over the antibody mediated response (Tuberculosis).
- Allowing the analysis of many samples in a relatively short time (2 days).

**Drawbacks**

- Samples must be analysed within 8h to avoid IFN-γ degradation (Rothel et al. 1992).
- Cross-reaction due to similar protein antigens (e.g., M. bovis and M. avium).

Disagreement about test performance:

- Different methods for interpretation of results
- Different criteria to determine the cut-off
- Absence of Local Gold Standard

Commercial tests are not always properly validated under local conditions.
Specific Bovine Brucellosis Diagnosis Based on In Vitro Antigen-Specific Gamma Interferon Production

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Received 6 June 1994/Returned for modification 1 September 1994/Accepted 29 November 1994

In order to improve the specificity of the diagnosis of bovine brucellosis, we developed a test which can be regarded as an in vitro correlate of the delayed-type hypersensitivity test (DTH). A mixture of cytoplasmic proteins from Brucella melitensis B115 was used as a specific antigenic stimulus in bovine whole blood culture. Supernatants harvested at 18 to 24 h after the in vitro antigenic stimulus were assayed for their gamma interferon (IFN-γ) content by using a commercial sandwich enzyme-linked immunosorbent assay kit. The IFN-γ assay was evaluated with 10 heifers during the course (80 days) of an experimental infection and with 14 cows from an ongoing brucellosis outbreak. All of these animals were slaughtered, and pertinent organs were subjected to classical bacteriological analyses. In addition, we analyzed 23 field cases in which false-positive serological reactions occurred. The IFN-γ results were compared with those of the standard DTH and a battery of serological assays, and they were correlated with bacteriological data. Both for the experimental infection and for the field brucellosis outbreak, the IFN-γ assay detected infection in more animals than any combination of the serological tests, and it detected infection earlier than these tests. Finally, none of the samples from cows showing false-positive serological reactions was classified as positive by the IFN-γ assay, attesting to its specificity and to its usefulness in interpreting ambiguous serological results. A rapid and convenient alternative to the DTH, the IFN-γ assay appears to be an ideal method that is complementary to the serological diagnosis protocols.

Experimental data with a reduced number of samples ——

No field studies
Field comparison of the interferon-gamma assay and the intradermal tuberculin test for the diagnosis of bovine tuberculosis

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SUMMARY: An extensive field comparison of the gamma Interferon (IFN-γ) assay and the single intradermal tuberculin test for the diagnosis of bovine tuberculosis was conducted in Australia. The specificity of the IFN-γ assay was determined by testing more than 6000 cattle from tuberculosis-free herds and varied from 96.2% to 98.1%, depending on the cut-off point chosen to define a positive reactor. For the sensitivity trial, cattle from herds being de-populated because of bovine tuberculosis were examined with both assays. The sensitivity of the IFN-γ assay was shown to be significantly higher than the single intradermal tuberculin test and varied from 76.8% to 93.6% depending on the method of interpretation. A maximum overall sensitivity of 95.2% was obtained by testing with the IFN-γ and the tuberculin test in parallel. The superior sensitivity of the IFN-γ assay and the ability to adjust the sensitivity of the system depending on the task involved, will provide the Australian Tuberculosis Eradication Campaign with a valuable additional test to enable it to accomplish its goals.

Aust Vet J 68: 286 – 290
Comparison of the sensitivity of the caudal fold skin test and a commercial γ-interferon assay for diagnosis of bovine tuberculosis

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“Sensitivity of the γ-INF assay ranges from 55.4 to 97.1%, depending on the standard of comparison and on the method of interpretation”.

<table>
<thead>
<tr>
<th>Using the same method and cut-off for interpretation of results</th>
<th>Sn</th>
<th>Sp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia (Wood et al, 1991)</td>
<td>93.6</td>
<td>96.2</td>
</tr>
<tr>
<td>E.E.U.U (Whipple et al, 1994)</td>
<td>73.0</td>
<td>Not done!</td>
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Field conditions and immunological background can be quite variable among different populations.
In vivo Delayed Type Hypersensitivity (DTH) reaction

When the allergen (proteins or peptides) is intradermically inoculated in an infected (sensitized) animal, macrophages are immobilized in the area and release enzymes causing:

- Local edema
- Granulomatous inflammation
- Vascular thrombosis and necrosis.

Method:

Intradermal injection of an allergen (Tuberculin, Brucellin, viral peptides..) in the caudal tail fold, neck or lower eyelid.

After 48-72 hours the site is examined for signs of swelling.
DTH reaction in Brucella infected cow

DTH reaction in Brucella infected sheep

Skin test
Skin test

DTH reaction in Brucella infected pigs
Useful when false positive serological reactions (FPSR), due to other common antigens (like lipopolysaccharides), are present.

**S-LPS/O-PS share** *Y. enterocolitica* O:9 epitopes

**CYTOSOLIC-PERIPLASMIC PROTEINS (BRUCELLIN)**
- Are mostly shared by *Brucella* species, and in contrast to S-LPS, they elicit DTH immunoresponses
- Are fully (mostly) genus-specific, and not shared with *Y. enterocolitica* O:9

**can solve the problem of FPSR**

**in vitro tests**
- IL/IFNg
- poor results

**in vivo**
- Skin test (DTH) (herd level)
Histopathological reaction should be further studied...

**HIPERSENSITIVITY TYPE IV - DTH**
(cell mediated response)

Mixed hypersensitivity reaction (Arthus +DTH) has been observed in pigs

**HIPERSENSITIVITY TYPE III - ARTHUS**
(antibody mediated response)

Large infiltrates of mononuclear cells

Hyperemia and perivascular infiltrates
Possible drawbacks to be taken into account...

- Animals have to be manipulated twice

- Interference with the immune status of the animal after injection:
  - Anergy period
  - Sensitization
Anergy state produced by the allergen

Local macrophages and T-cell remain inactive during a certain period after the DTH reaction.

Anergy periods upon inoculation with Tuberculin or Brucellin allergens:

Tuberculin in cows: 60 days

Brucellin in cows: unknown

Brucellin in sheep: 24 days

Experimental data (Blasco et al, 1994)

Brucellin in pigs: NO anergy phenomenon observed.

*Brucella* infected pigs showing positive reaction in DTH test were grouped (n 12) and submitted to a second DTH test (same allergen and dose) at regular intervals (1, 2, 3, 4 and 6 weeks) after the first inoculation. All pigs gave positive reaction after the second inoculation regardless of the elapsed time from the first one.
Sensitization produced by the allergen

Some allergens elicit the activation of macrophages and T-cells by themselves, leading to undesirable DTH positive reaction in non infected animals. Allergens must be free of sensitizer molecules.

Undesirable DTH reactions reported when using *Brucella* allergens containing hydrolitic polysaccharides (Alton G.C. Animal Brucellosis. CRC Press). No sensitization problems reported using *Brucella* citosolic proteins (Brucellin free of polysaccharide).

Purified protein derivative (PPD) products replaced heat-concentrated synthetic medium tuberculins.
Possible drawbacks to be taken into account...

- Animals have to be manipulated twice

- Interference with the immune status of the animal after injection:
  - Anergy period
  - Sensitization

- Interferences in serological tests
  - Not necessarily when using different antigens

- Cross-reaction due to similar protein antigens
Interferences in serological tests

RBT  CFT  ELISA

Serological tests applied for Brucellosis routinary diagnostic are based in surface smooth antigens (S-LPS / O-PS)

The use of cytosolic extracts from rough strains (free from O-PS precursors) prevent serological interference
**Cross-reaction due to similar protein antigens**

The comparative intradermal tuberculin test with bovine and avian PPD is used to differentiate between animals infected with M. bovis and those sensitised due to exposure to other mycobacteria or related genera.

Cross-reaction between *Brucella* and phylogenetic neighbors (*Ochrobactrum*) citosolic proteins has been experimentally demonstrated, but cause no significant trouble in field diagnostic.
Possible drawbacks to be taken into account...

- Animals have to be manipulated twice

- Interference with the immune status of the animal after injection:
  - Anergy period
  - Sensitization

- Interferences in serological tests
  - Not necessarily when using different antigens

- Cross-reaction due to similar protein antigens

- Lacks specificity after vaccination
  - Allergic reactions detected in vaccinated cows several years after vaccination with all Brucella vaccines
Routinary diagnostic techniques have to be...

100% specific (ideally)

Diagnostic sensitivity as high as possible

As EASY as possible

As CHEAP as possible

And above all...Well validated!!!

Diagnostic performance and cut-offs should be always determined using LOCAL Gold Standard populations
Message to take home...

Diagnostic laboratory

Director of Control and Eradication strategies against animal infectious diseases

Thanks for your attention!!

[Images and diagrams related to diagnostic laboratories and space travel, with text on messages and a logo for CITA.]