In vitro Diagnosis of Drug Allergy: Current Status and Perspectives

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Drugs as Immunogens

- Biologics: foreign macromolecules (*e.g.* antibodies, recombinant proteins) act directly as immunogen
- Drugs (non-biologics)
  - Hapten – drug (*e.g.* β-lactam antibiotics, quinidine) combines with a host macromolecule
  - Pro-hapten – processed drug (*e.g.* sulfonamides, phenytoin) combines with a host macromolecule
- Drugs can act directly to stimulate an immune receptor (pharmacologic interaction with immune receptors = p-i concept)

Use of *in vitro* Testing for Drug Allergy

- Testing in the setting of an immediate drug reaction
- Testing in the setting of a delayed drug reaction
- Testing on the horizon

Immediate Reaction to Drug

- Gell and Coombs type 1 reaction that occurs rapidly upon exposure to a specific drug
- Standard approach to evaluate is immediate skin testing (penicillin major and minor determinants are validated, other drugs ?)
- In vitro methods of evaluation include:
  - Tryptase to establish mast cell degranulation
  - Allergen (drug) specific IgE testing
  - Basophil activation test (BAT)

Tryptase Testing

- Mature tryptase reflects mast cell degranulation and is elevated in a systemic allergic reaction
- Current laboratory test most widely available measure total tryptase (not mature tryptase)
  - Released within 30-60 minutes following activation and half life is ~2 hours allows longer “testing window”
  - Levels above normal range (vary among labs: 10-11.4 ng/mL) are consistent with anaphylaxis (or increased mast cell numbers) but the sensitivity is not high
  - More sensitive test for anaphylaxis: mature tryptase level or a total tryptase rise over baseline of > 2 ng/mL

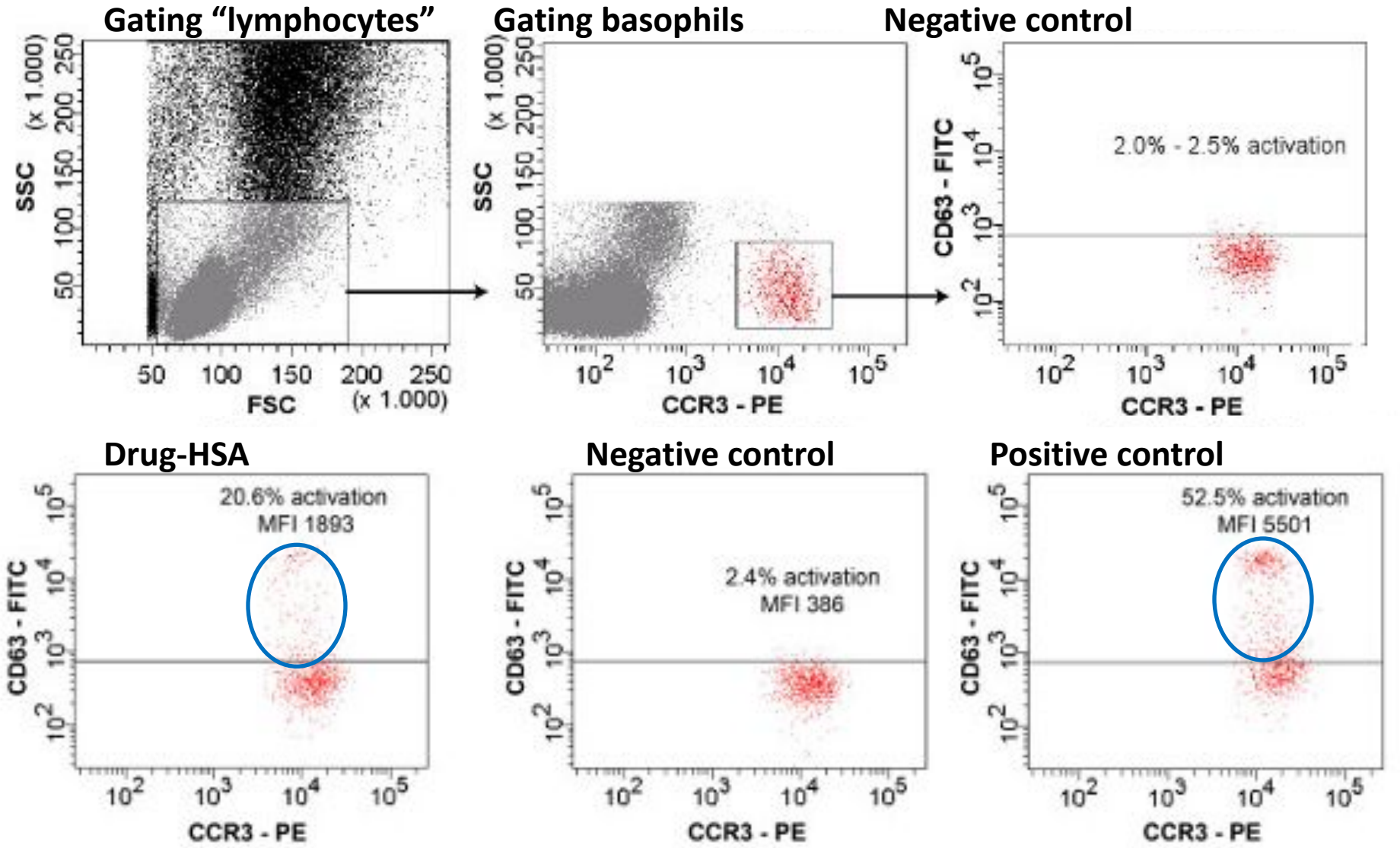
Allergen Specific IgE Testing

- *In vitro* "equivalent" of immediate skin testing
- Does not subject patient to risk and does not have a potential of inducing sensitization
- Limited range of drugs available impacts utility: β-lactams (penicilloyl G & V, ampicilloyl, amoxocilloyl), ACTH, cefator, ceftriazone, chlorhexidene, ethylene oxide, gelatin, insulin, neuromuscular blocking agents, tetanus toxoid)
- Tests generally have high specificity with lower sensitivity - **negative test does not rule out allergy**

Basophil Activation Test

- Test evaluates basophils present in either whole blood or separated mononuclear cells
- Validated for aeroallergens, hymenoptera venoms, foods, latex, some drugs (generally based on a generated drug-protein complex)
- Commercial assay (not FDA approved in USA): uses expression of CCR3 to identify basophils and expression of CD63 to identify activation after incubating cells the with drug complex
- “Enhanced assay” adds a third marker, CD203c

Basophil Activation Test



Positive control: 52.5% CD63+, SI - 5501/386 = 14.2

Positive drug BAT: 20.6% CD63+; SI - 1893/386 = 4.9

Steiner, M. et al. J Vis Exp 2011

Basophil Activation Test

- Advantages
  - Does not subject patient to any risks
  - Functional test that resembles the *in vivo* pathway
  - Relatively good sensitivity with high specificity
  - Positive BAT depends on type of allergen
    - Aeroallergens/foods $\geq$15% CD63+ basophils
    - Venoms $\geq$10% CD63+ basophils
    - Drugs (β-lactams, analgesics) $\geq$5% CD63+ basophils
- Disadvantages
  - Must have viable, non-activated cells (24 hr "window")
  - More limited availability since it requires a flow cytometer and generation of drug-protein (hapten-carrier) complex
  - **Negative test does not rule out drug allergy**

BAT in Radiocontrast Media Reactions

- Evaluation of 26 patients with history of immediate radiocontrast media (RCM) reactions: BAT using five different RCM products (tested months later)
- BAT results: 15/26 patients had a positive BAT
  - 1:100 RCM: patients = 13.1% CD63+/SI=8.1 (p=0.01)
    controls = 2.7% CD63+/SI=1.5
  - 1:10 RCM: patients = 19.2% CD63+/SI=9.0 (p=0.001)
    controls = 3.7% CD63+/SI=2.3
- Receiver Operator Curve (ROC) area under the curve was 0.79 = test with moderate accuracy

Pinnobphun P, et al. Ann Allergy Asthma Immunol 2011, 106:387

Delayed Immunologic Reaction to Drugs

- Most commonly linked to cellular response (Gell and Coombs Type IV reaction involving T cells)
- These reactions have been subdivided into
  - Type IVa: mediated by Th1 response
  - Type IVb: mediated by Th2 response
  - Type IVc: mediated by cytotoxic cell response
  - Type IVd: mediated by neutrophilic inflammation
- Additional data now suggests that some reactions involve conventional TcR activation (*e.g.* where there is an HLA link) and others involve direct drug-immune receptor interaction (p-i concept)

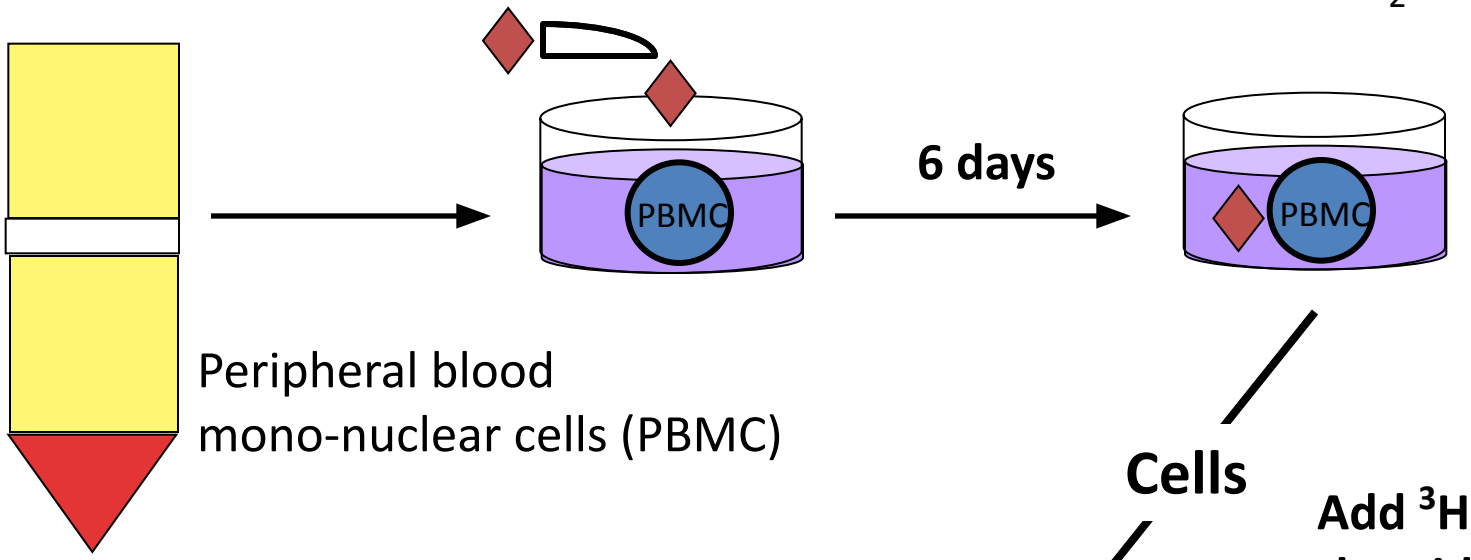
Focus of *in vitro* Testing

- Confirm that the clinical findings are the result of an immunologic response (rather than a pharmacologic or idiosyncratic response)
- Identify the causative drug in settings where multiple drugs have been administered
- Current testing methods
  - Lymphocyte transformation test (LTT)
  - CD69 upregulation flow cytometry test
  - Cytokine production
  - Evaluation of cytotoxicity (or its products)

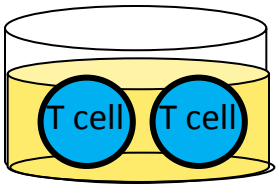
Lymphocyte Transformation Test (LTT)

I- Activation *in vitro*

Varied concentrations of pure drug, incubate at 37ºC with 5% CO_2



II- Quantify Response



Harvest cells and count radioactivity, results: cpm or stimulation index (SI = drug stimulated cpm/unstimulated cpm)

Lymphocyte Transformation Test (LTT)

- Must use controls to establish lack of drug induced toxicity and to rule out non-specific activation
- Must have viable cells and requires sterile tissue culture
- LTT has been successfully applied to drug associated:
  - Maculopapular exanthem
  - Pustular exanthem
  - Stevens Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN)
  - Drug rash with eosinophilia and systemic symptoms (DRESS)
- Positive LTT has generally been defined as a stimulation index (SI = cpm with drug/cpm with medium) > 2
- Sensitivity is 60-70% under optimal conditions with a higher specificity
- **Negative test does not rule out T cell mediated drug response**

Evaluation of LTT in Different Types of Delayed Hypersensitivity Drug Reactions

- 27 patients in three groups: 8 maculopapular eruptions (MP), 6 SJS + 2 TEN, 11 DRESS
- Evaluated by LTT at 1 week, 2-4 weeks, 5-8 weeks, 1 year and > 1 year following onset
- Patients with MP and SJS/TEN had positive LTT at 1 week post-onset, response declined over time
- Patients with DRESS were negative at 1 week and were positive at 5-8 weeks

Kano Y, et al. Allergy 2007, 62:1439

LTT Used to Identify the Drug that Induced DRESS

- Two patients receiving multiple drugs including anticonvulsants and antibiotics associated with the development of DRESS
- Evaluation by LTT utilized all drugs that had been given, each at 7 concentrations (1-200 μg/ml)
- Studied 3 months after the clinical presentation
- Causative drug was identified as ceftriaxone in one pt and piperacillin-tazobactam in the other pt
- LTT assay proved valuable in defining the drug associated with DRESS (avoid in the future)

Jurado-Palomo J, et al. J Investig Allergol Clin Immunol 2010, 20:433

LTT Summary

- LTT appears to be a suitable complement to other testing in delayed drug reactions
- Time line of positivity may differ between the different types of delayed drug reactions
- Positive test helps identify the offending drug but a **negative test does not rule out drug related hypersensitivity**
- The test remains a research tool, it is not standardized and it requires tissue culture with results available after six or more days

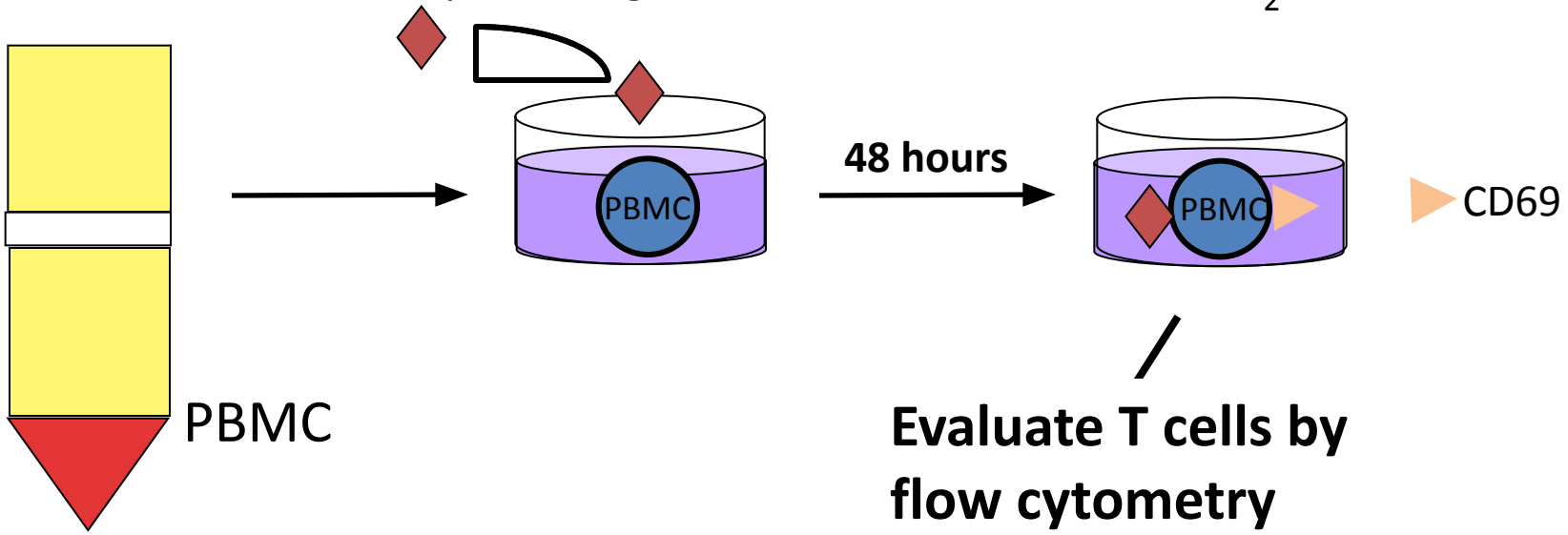
Alternatives to LTT (^{3}H Thymidine)

- Evaluation of upregulation of a T cell activation antigen in response to *in vitro* drug exposure
  - CD69 up-regulation, an early product of T cell activation, measured by flow cytometry at 48 hrs
- Ex vivo cytokine production
  - Cytokine secretion into the supernatant following mononuclear cell culture with drug (*e.g.* γ-IFN)
  - Elispot assay measures individual T cell production of a cytokine following *in vitro* drug stimulation

T cell CD69 Upregulation

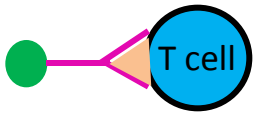
I- Activation *in vitro*

Varied concentrations of pure drug, incubate at 37ºC with 5% CO_2



Evaluate T cells by flow cytometry

II- Quantify Response



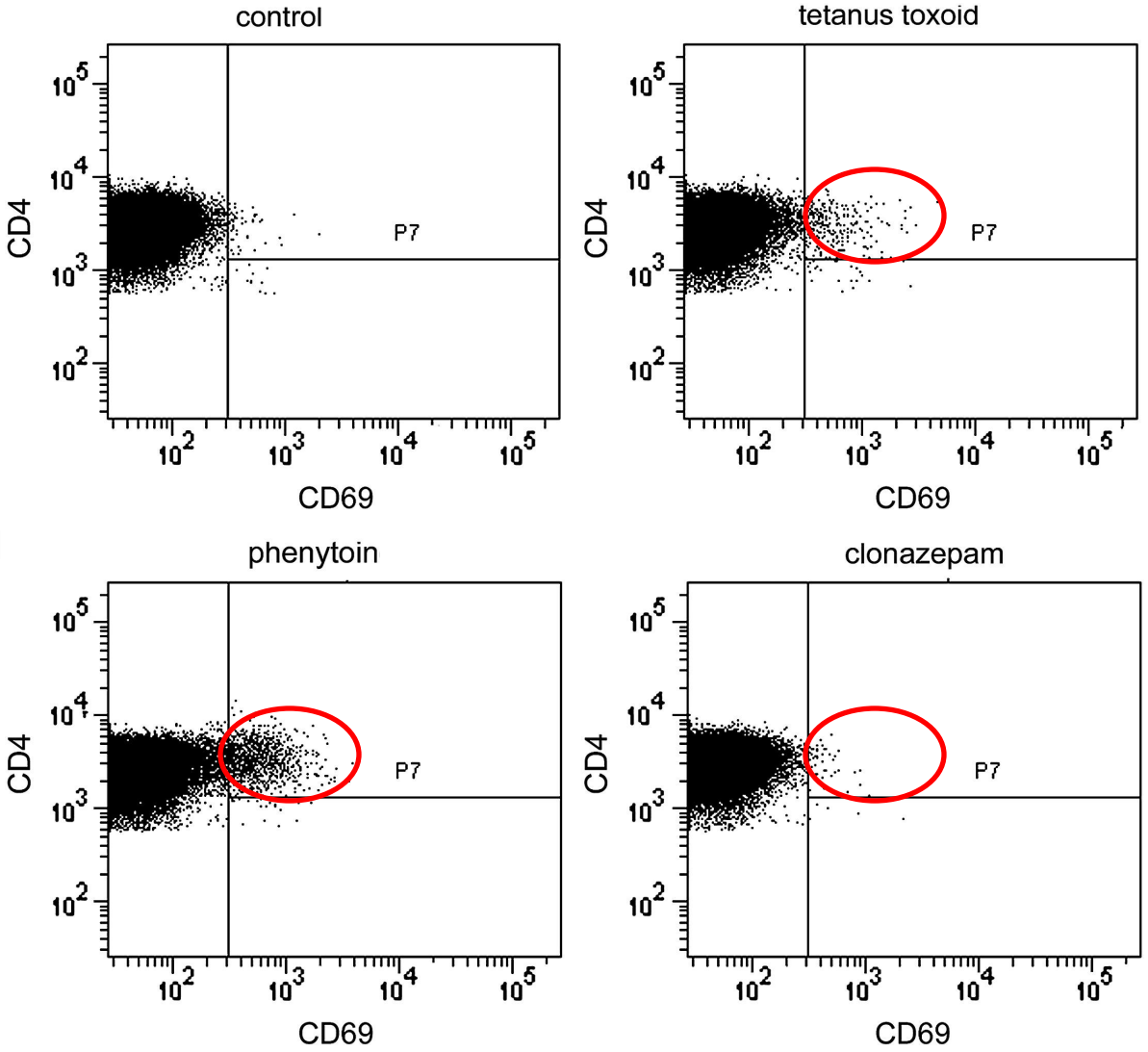
CD69 upregulation expressed as percent CD69 positive T cells

CD69 Upregulation in Response to Drug

Evaluation of a phenytoin-allergic patient following 48 hrs of stimulation
medium - negative control
Tetanus toxoid - positive control

Phenytoin - positive test

Unrelated drug clonazapam – negative test



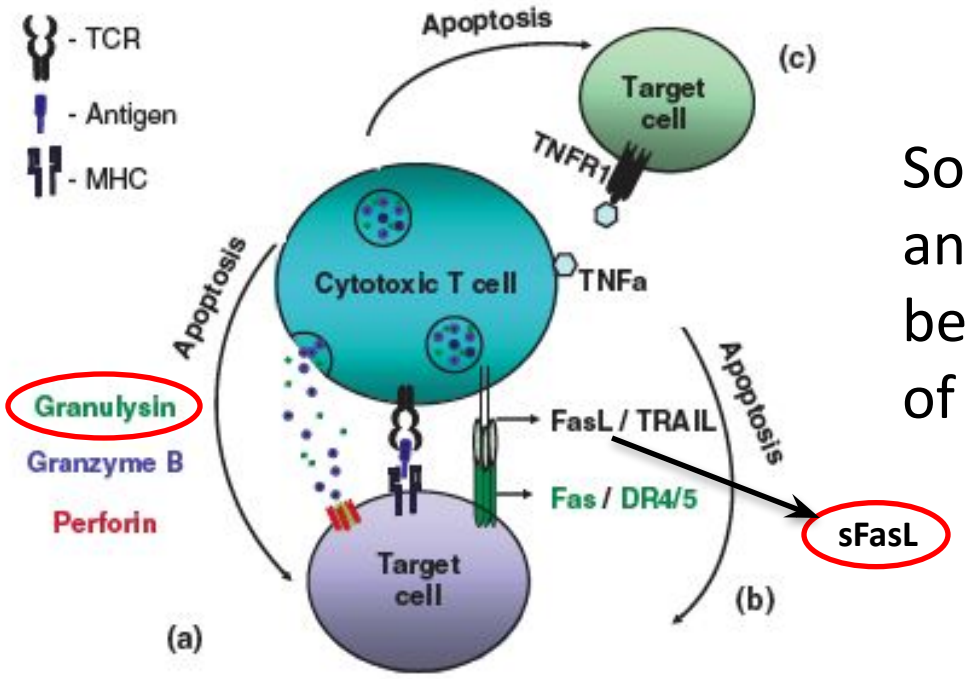
Lochmatter P, et al. Immunol Allergy Clin N Am 2009, 29:537

Summary of LTT Alternatives

- CD69 upregulation appears to perform similar to LTT with the advantage of being a 48 hour assay and not requiring radionuclides
- Cytokine production assays correspond to LTT but the actual cytokine produced does not appear to correlate well with the clinical phenotype (*i.e.* IFN-γ is typically produced with all types of delayed drug reactions)

Immunopathogenesis of SJS/TEN

Bullous skin processes (SJS/TEN) associated with drugs appear to be linked to cytotoxic T cell activity



Soluble Fas ligand (sFasL) and granulysin have been found in the serum of patients with SJS/TEN

Porebski G, et al. Clin Exp Allergy 2011, 41:461

“Real Time” Test to Diagnose SJS/TEN

- The serum level of granulysin is ~100X greater than sFasL in SJS/TEN making it an attractive target
- An immunochromagraphic test for serum granulysin (≥10 ng/mL) predicted SJS/TEN 2-4 days prior to mucocutaneous reuptions
- This assay could prove useful in predicting when a drug reaction will lead to SJS/TEN

Fujita Y, et al. J Am Acad Dermatol 2011, 65:65

In the Future

- Multiplex cytokine evaluation following in vitro culture (e.g. IFN-γ, IL-2, IL-4, IL-5, IL-8, IL-13, IL-17, etc) may reveal specifics about the type of immune response
- Nature of drug derived epitopes inducing an immune reaction often are not well understood
  - Mass spectrometry (MS) has evolved as a powerful tool to evaluate proteomics and metabolomics
  - MS used to characterize the functional antigens derived from piperacillin (in CF patient serum) with the identification of multiple drug derived haptenic structures bound to albumin
    (Whitaker P, et al. J Immunol 2011, 187:200)

Summary *in vitro* Testing in Drug Allergy

- Immediate drug reactions
  - Specific IgE testing: safe test but there are limited numbers of suitable drug conjugates available for testing
  - BAT: promising functional test that requires viable cells and a drug conjugate preparation for activation
- Delayed drug reactions
  - Lymphocyte transformation test (LTT)
    - Most common research method to determine responsible drug
    - Issues remaining include: standardization, requirement for viable cells, six day sterile tissue culture period and use of radionuclides
  - CD69 upregulation may be equivalent to LTT – under study
  - *In vitro* cytokine production to drug – under study
  - Product of cytotoxic cells (granulysin) promising to help dx SJS/TEN prior to mucocutaneous symptoms (further study)

Conclusions

- The clinical story remains the most important starting point evaluating possible drug allergy
- *In vitro* testing can be complementary to *in vivo* testing and is evolving for the evaluation of both immediate and delayed drug allergy
- There is currently no single laboratory test that reliably establishes the drug responsible for an immunologically mediated drug reaction

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