

Technical Report

Development of a Delayed-Type Hypersensitivity (DTH) Model in the Cynomolgus Monkey

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Abstract: Although a T-dependent antibody response (TDAR) assay is generally recommended as the first-line immune function assay in nonclinical immunotoxicity evaluation, second-line assays such as delayed-type hypersensitivity (DTH) to measure cell-mediated responses can provide helpful additional information. In this study, male Cynomolgus monkeys were injected intramuscularly either once or twice with 1 mg Keyhole Limpet Hemocyanin (KLH) or twice with a commercially available tetanus vaccine (40 IU tetanus toxoid + 0.06 mg aluminum hydroxide). All animals were subsequently challenged by intradermal injections of the same antigen or aluminum hydroxide after 4, 6 and 8 weeks. Clinical reactions at the injection sites were scored 24, 48 and 72 h post challenge. Skin biopsies were taken on completion of the observation period after each challenge for standard histological examination and immunolabeling using CD3 (T lymphocytes), CD19 (B lymphocytes) and CD68 (macrophages) antibodies. Tetanus toxoid induced stronger clinical reactions than KLH, whereas aluminum hydroxide induced no clinical reaction. Perivascular mononuclear cell infiltrates, a histopathological finding consistent with a DTH reaction, were seen after all challenges with tetanus toxoid or KLH, but not with aluminum hydroxide. Immunohistochemistry evidenced the presence of T lymphocytes and macrophages within these infiltrates. These results suggest that tetanus toxoid adjuvanted with aluminum hydroxide can induce a consistent DTH response for use as a model of cell-mediated response in Cynomolgus monkeys. (DOI: 10.1293/tox.25.183; J Toxicol Pathol 2012; 25: 183–188)

Key words: immunotoxicity evaluation, delayed-type hypersensitivity, cynomolgus monkeys, tetanus toxoid, KLH

Introduction

Nowadays a T-dependent antibody response (TDAR) assay is widely considered to be the first-line function assay when the weight of evidence review as defined by the ICH guideline S8¹ recommends that additional immunotoxicity studies should be performed to assess the immunotoxic potential of drug candidates. Nevertheless, second-line function assays may be further needed case-by-case depending on histopathological and clinical findings in standard toxicity studies, the results of the TDAR assay or the drug's mechanism of action. In addition to lymphocyte subset analysis (immunophenotyping) and assays to measure natural killer (NK) cell activity or neutrophil/macrophage function, it may be helpful to assess cell-mediated immune responses using either *in vitro* or *ex vivo* assays (e.g., lymphoprolifera-

tion induced by mitogens or mixed lymphocyte reaction) or *in vivo* animal models.

Although assays to measure cell-mediated immunity have long been used, especially in rodents^{2–4}, they are rather seldom included in current nonclinical immunotoxicity evaluation. One reason may be that only limited efforts have been paid to standardize and validate these assays until recently. The situation, however, is evolving, as shown by the recent study in B6C3F1 mice by Smith and White⁵. In comparison to *in vitro* assays, *in vivo* models of cell-mediated immunity offer the advantage of measuring multiple cellular components involving several cell interactions, inflammatory mediators and complex signaling cascades. Thus, they can be useful for assessing cell-mediated immunity as well as general immune competence.

Nonhuman primates (NHP) are often the only relevant species available for the nonclinical safety evaluation of novel biopharmaceuticals because of increasingly species-specific targets⁶. So far, only few studies have been devoted to designing *in vivo* models of cell-mediated immunity in NHP^{7–9}. The aim of the present study was to develop a delayed-type hypersensitivity (DTH) model in the Cynomolgus monkey for use in regulatory immunotoxicity evaluation.

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Materials and Methods

Animals

Male purpose-bred Cynomolgus monkeys (*Macaca fascicularis*) purchased from Noveprim Europe (Ebene, Mauritius) were used throughout this study. The animals were housed in individual stainless steel cages in a dedicated primate unit where room conditions were set as follows: temperature, $24 \pm 3^\circ\text{C}$; relative humidity, $50 \pm 30\%$; light/dark cycle, 12h/12h (7:00–19:00); ventilation, approximately 12 cycles/h of filtered, non-recycled air. Enrichments were given to the animals during the whole study. All animals had free access to tap water and were distributed approximately 180 grams of OWM (E) SQC SHORT expanded diet (Dietex France, SDS, Saint Gratien, France). In addition, a fruit supplement was given daily to each animal. They were at least 3 years old at the beginning of the study.

All study procedures were conducted according to a written study protocol approved by CiToxLAB animal ethics committee and facility standard operating procedures in strict compliance with accepted animal welfare standards.

Treatment

The animals were allocated to 3 groups of 3 animals each. They were first immunized by the intramuscular route and subsequently challenged by intradermal injections.

Immunization: Group 1 and 2 animals were injected intramuscularly with 1 mg of keyhole limpet hemocyanin (KLH), purchased from Thermo Scientific (France) and prepared as a 2 mg/mL solution in water for injection, either on day 14 (group 1) or on days 1 and 14 (group 2). Group 3 animals were injected on days 1 and 14 with 0.5 mL of a tetanus vaccine (Vaccin Tétanos Pasteur®) purchased from Sanofi-Pasteur Laboratories (France), corresponding to a dose of 40 UI tetanus toxoid plus 0.6 mg aluminum hydroxide.

Challenges: On the days of challenge, all animals were fasted at least 12 h before anesthesia with intravenous propofol (Rapinovel®, B Braun Medical). The backs (and abdomen when appropriate) of the animals were carefully clipped free of hair with electric clippers under anesthesia. Group 1 and 2 animals received 3 intradermal injections of 0.05 mg/mL of keyhole limpet hemocyanin (KLH) on the back on days 42, 56 and 70 (3 challenges per animal and per day of challenge for a total of 9 challenge sites per animal). Group 3 animals received 3 intradermal injections of 0.05 mg/mL of the same tetanus vaccine and 3 injections 0.05 mg/mL of a 1.2 mg/mL solution of aluminum hydroxide on days 42, 56 and 70, either on the back or on the abdomen of the animals (3 challenges per test item, per animal and per day of challenge for a total of 18 challenge sites per animal). The injection sites were disinfected with 70% alcohol prior to intradermal injection, and each injection site was used only once.

Delayed-type hypersensitivity (DTH) response

The DTH response of each monkey was assessed both clinically and histologically.

Clinical examination: Skin reactions at the intradermal injection sites were recorded once daily for each animal by visual inspection and palpation of the injection sites prior to injection and then after 24, 48 and 72 h. The following scales were used for scoring erythema formation (very slight, barely perceptible = 1; slight = 2; moderate = 3; severe erythema to slight eschar formation = 4) and edema formation (very slight, barely perceptible = 1; slight with area edges well-defined by definite raising = 2; moderate with approximately 1-mm of raising = 3; severe with more than 1-mm of raising and extension beyond area of exposure = 4). Any other lesions, such as abscess, necrosis and local inflammation, or reactions, such as pain, were recorded.

Histopathological and immunohistochemistry examinations: Skin biopsies were collected under propofol anesthesia 72 h after intradermal injection of the designated challenge sites using an 8-mm skin biopsy punch. Prophylactic analgesia was provided by subcutaneous injection of buprenorphine (Buprecare®) after each challenge. Three skin biopsies (groups 1 and 2) or 6 skin biopsies (group 3: 3 skin biopsies following tetanus toxoid challenges and 3 following aluminum hydroxide challenges) were collected after each day of challenge from each animal. Two out of 3 biopsies were fixed in 10% buffered formalin and then embedded in paraffin wax, sectioned at a thickness of approximately 4 microns and stained with hematoxylin and eosin (2 sections per site). The third biopsy was snap frozen embedded in OCT (Optimal Cutting Temperature) compound in a plastic mold, frozen in dry ice and stained with an immunoperoxidase technique using CD3 (T lymphocytes), CD19 (B lymphocytes) and CD68 (macrophages) commercial markers.

A microscopic examination was performed by Dr Gervais on all skin biopsies from all animals (including sections stained with hematoxylin and eosin or labeled for immunohistochemistry). A peer review was performed by Dr Fleurance on an adequate number of slides from challenge sites to confirm that findings recorded by the study pathologist were consistent and accurate.

Results

No unscheduled deaths occurred, and no systemic treatment-related clinical signs were reported. Body weight was never affected during the study.

Clinical DTH response

Local reactions at injection sites were observed in all groups after each challenge, generally during 2 or 3 days. In groups 1 and 2, erythema (mean grades = 0.2 to 0.9) and/or thickening (mean grades = 0.3 to 1.1) at the injection sites were observed 24, 48 and 72 h after the first KLH challenge on day 42. After the second KLH challenge on day 56, erythema was seen until 48 h after injection (mean grades = 0.4 to 1.1) and was associated with thickening at all 3 time-points (mean grades = 0.3 to 0.9). No erythema was recorded after the third KLH challenge on day 70, but edema was

Table 1. Summary of Main Local Reactions Indicative of a DTH Response Following Challenge with Either KLH or Tetanus Vaccine in Previously Immunized Cynomolgus Monkeys (Mean Grading and Incidence^{a)})

Groups	1			2			3					
	One KLH immunization			Two KLH immunizations			Two tetanus vaccine immunizations					
Hours after challenge	24	48	72	24	48	72	24	48	72	24	48	72
<i>First challenge (Day 42)</i>	KLH			KLH			Tetanus vaccine			Aluminium hydroxide		
Erythema	0.9 6/9	0.3 3/9	0.3 3/9	0.7 6/9	0.2 3/9	0.2 2/9	1.3 7/9	1.1 6/9	1.1 6/9	0 0/9	0 0/9	0 0/9
Thickening	1.1 9/9	1.1 9/9	1.1 9/9	0.3 3/9	0.3 3/9	0.3 3/9	0.8 5/9	1.2 6/9	1.3 6/9	0 0/9	0 0/9	0 0/9
Edema	0 0/9	0 0/9	0 0/9	0 0/9	0 0/9	0 0/9	0.2 2/9	0.2 2/9	0.2 2/9	0 0/9	0 0/9	0 0/9
<i>Second challenge (Day 56)</i>	KLH			KLH			Tetanus vaccine			Aluminium hydroxide		
Erythema	0.4 4/9	0.7 6/9	0 0/9	1.1 7/9	1.1 7/9	0 0/9	1.2 8/9	1.3 8/9	1.1 7/9	0 0/9	0 0/9	0 0/9
Thickening	0.8 7/9	0.8 7/9	0.3 3/9	0.9 8/9	0.9 8/9	0.3 3/9	1.3 9/9	1.7 9/9	1.2 7/9	0 0/9	0 0/9	0 0/9
Edema	0 0/9	0 0/9	0 0/9	0 0/9	0 0/9	0 0/9	0 0/9	0.2 2/9	0.2 2/9	0 0/9	0 0/9	0 0/9
<i>Second challenge (Day 70)</i>	KLH			KLH			Tetanus vaccine			Aluminium hydroxide		
Erythema	0 0/9	0 0/9	0 0/9	0 0/9	0 0/9	0 0/9	1.3 6/9	1.3 6/9	0.2 1/9	0 0/9	0 0/9	0 0/9
Thickening	0 0/9	1 9/9	0 0/9	0 0/9	1 9/9	0 0/9	0 0/9	1 9/9	0.7 6/9	0 0/9	0 0/9	0 0/9
Edema	1 9/9	0 0/9	0 0/9	1 9/9	0 0/9	0 0/9	1 9/9	0 0/9	0 0/9	0 0/9	0 0/9	0 0/9

^{a)} Results expressed as incidences with numbers of animals bearing lesions per number of animals examined.

noted at all the injection sites 24 h after injection, and then thickening was observed 48 h after injection. Similar local reactions were noted in group 1 animals immunized with one KLH injection and in group 2 animals immunized with two KLH injections.

In group 3, local reactions including erythema and/or thickening, sometimes associated with edema at the injection sites, were observed 24, 48 and 72 h after the first challenge with tetanus vaccine on day 42 (mean grades = 1.1 to 1.3 for erythema and 0.8 to 1.3 for thickening) and after the second challenge on day 56 (mean grades = 1.1 to 1.3 for erythema and 1.2 to 1.7 for thickening). Erythema was recorded 24, 48 and 72 h after the third challenge on day 70 (mean grades = 0.2 to 1.3), edema was recorded 24 h after injection, and then thickening was recorded 48 and 72 h after injection. Importantly, no local reactions were observed on any occasion after aluminum hydroxide challenges on days 42, 56 and 70, indicating that tetanus toxoid instead of aluminum hydroxide was the causative factor of observed local reactions induced by tetanus vaccine.

Although interindividual variations and intersite variations for the same animal were noted after challenge with both KLH and tetanus vaccine, local reactions after tetanus vaccine injections consistently had a greater score than local

reactions after KLH injections (Table 1).

Histopathology and immunohistochemistry findings

The inflammatory responses elicited by intradermal challenge with 0.05 mg/mL of KLH on days 42, 56 and 70 on the backs of the 3 monkeys immunized on day 14 (group 1) and the 3 monkeys immunized on days 1 and 14 (group 2) were comparable across individuals and consisted of perivascular mononuclear inflammatory cell infiltrates (Fig. 1). This infiltrate was comprised of CD3⁺ and CD68⁺ cells corresponding to T lymphocytes and macrophages. There were no CD19⁺ lymphocytes (Fig. 2). Similar findings were observed with tetanus toxoid (group 3). Granulocytes were also observed, especially in the vicinity of necrotic foci in the subcutis.

There were no clear differences between the animals previously immunized once (group 1) or twice (group 2) with KLH, but the reaction was more marked with tetanus vaccine (group 3), thus confirming clinical findings. In addition, no clear differences were identified between days 42, 56 or 70, and histological findings were still present on day 70. It is also noteworthy that there were interindividual variations (Table 2).

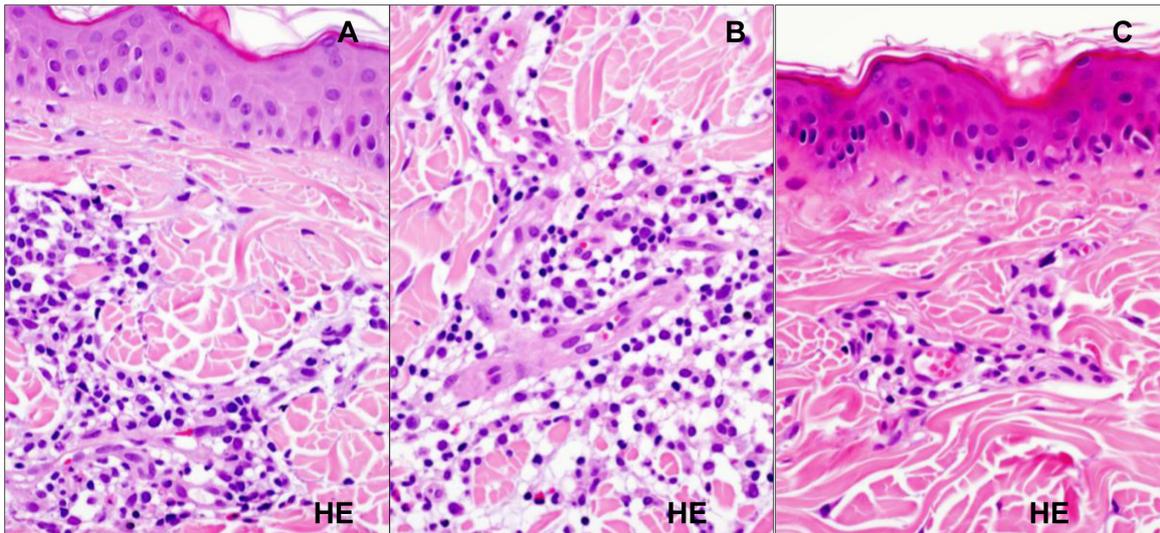


Fig. 1. Challenge sites on day 45 (hematoxylin and eosin, 200-fold magnification). (A) Perivascular mononuclear inflammatory cell infiltrates in a monkey immunized twice and challenged with KLH. (B) Perivascular infiltrates in a monkey immunized twice and challenged with tetanus vaccine. (C) Monkey injected with aluminum hydroxide.

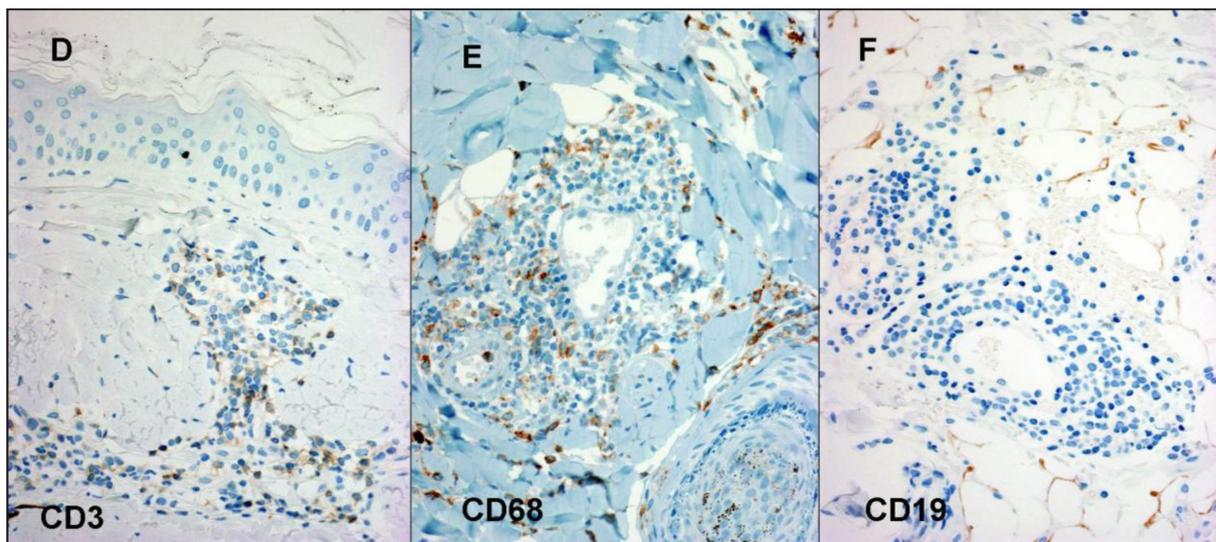


Fig. 2. Challenge sites on day 45 from a monkey immunized and challenged with KLH (200-fold magnification). (D) Positive brown staining for T lymphocytes (CD3⁺). (E) Positive brown staining for macrophages (CD68⁺). (F) Lack of specific staining with CD19 (B lymphocytes) in the perivascular infiltrates.

Discussion

In the present study, both KLH and tetanus toxoid were found to induce a DTH response in Cynomolgus monkeys following the required design including a sensitizing (immunization) phase and an eliciting (challenge) phase. KLH and tetanus toxoid were selected for this comparative study as they are often used for induction of DTH in humans^{10–12} as well as monkeys^{7,8,13,14}. Dinitrochlorobenzene (DNCB) has also been proposed for induction of cell-mediated immune response in monkeys^{15,16}. However, DNCB is a contact sensitizer, and different effector and control mechanisms

are involved in contact sensitivity vs. DTH¹⁷.

Although both KLH and tetanus toxoid induced DTH in the present study, a greater response was consistently achieved with tetanus toxoid, both clinically and histologically. Despite interanimal variability, erythema, edema and induration assessed 24–72 h post challenge were found to be reliable clinical criteria of a DTH response. A critical issue with DTH models in monkeys is the pattern of histological changes. Perivascular mononuclear inflammatory cell infiltrates consisting of T lymphocytes and macrophages were evidenced, and this supports the conclusion that either KLH or tetanus toxoid did induce a classical DTH response in the

Table 2. Summary of Main Microscopic Findings Following Challenge with Either KLH or Tetanus Vaccine in Previously Immunized Cynomolgus Monkeys (Mean Grading and Incidence; Grading: 1 = Minimal, 2 = Slight, 3 = Moderate, 4 = Marked)

Groups	1			2			3					
	One KLH immunization			Two KLH immunizations			Two tetanus vaccine immunizations					
Days of Biopsy (72 hours after each challenge)	45	59	73	45	59	73	45	56	70	45	56	70
Number of biopsies (a)	6	6	6	6	6	6	6	6	6	6	6	6
Challenge	KLH			KLH			Tetanus vaccine			Aluminium hydroxide		
Serocellular crust	1	-	1	-	-	1	1	4	2.8	1	-	1
- Incidence	1	-	2	-	-	1	1	1	5	1	-	1
Ulcer	2	-	-	-	-	-	3	3.5	3	-	-	-
- Incidence	1	-	-	-	-	-	1	4	4	-	-	-
Acanthosis	1	1	1	-	1	1	1.2	1.5	1.8	-	1	-
- Incidence	2	2	2	-	1	1	5	4	5	-	1	-
Granulocyte infiltrates	1.9	1.8	1.2	1.0	1.1	1	2.5	3	2	-	-	-
- Incidence	6	5	6	6	5	6	6	6	6	-	-	-
Mononuclear inflammatory cell infiltrates	2.2	1.8	1.5	1.5	2.2	1.5	2.7	3.7	3.2	1	1	1
- Incidence	5	5	6	6	5	6	6	6	6	3	2	2
Macrophage infiltrates	-	2	1	-	1	1	2.7	3	2.5	-	-	-
- Incidence	-	1	2	-	1	1	6	6	6	-	-	-
Degeneration/necrosis in subcutis	1	2	1	-	1	1	2.7	3	1	-	-	-
- Incidence	2	2	1	-	3	1	6	5	3	-	-	-

(a) Two biopsies per animal and per day for microscopic examination.

present study⁸. Price⁹ reported that a 2-week interval between immunization and elicitation was seemingly too short to induce typical DTH and that a 4-week interval would be required. In our experimental conditions, a 4-week interval was sufficient to induce DTH, and similar clinical and histopathological findings were observed after 6-week and 8-week intervals.

The pioneering NTP interlaboratory immunotoxicity validation study in B6C3F1 mice, which tested a battery of reference compounds using various endpoints, showed that DTH is a predictor of cell-mediated immunity comparable to the *in vitro* lymphocyte proliferation assay¹⁸. This is in agreement with earlier human data, which evidenced a good correlation between *in vitro* cell-mediated immune responses and DTH^{19–21}. Although DTH models have been developed and used since the early days of immunotoxicology^{2,3}, limited attention has been paid to these models until recently. One advantage of DTH models as compared with *in vitro* assays is the ability to assess signaling cascades and cell-mediated immune responses in a more complex setting involving cell interactions, inflammatory mediators and trafficking proteins. Another advantage is the possibility to evaluate dose-response relationships more reliably. In contrast, because this is an *in vivo* model, DTH requires satellite groups of animals, and this reduces its cost-effectiveness.

The results of the present study show that a classical DTH reaction can be induced in Cynomolgus monkeys with either KLH or tetanus toxoid, although tetanus toxoid produced a greater response. Therefore, the tetanus DTH model appears to be a valid alternative for further study when

monkeys are the relevant species for assessing the potency of immunopharmacological effects or the immunological safety of drug candidates.

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