

# Modified short-term guinea pig sensitization tests for detecting contact allergens as an alternative to the conventional test

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The conventional adjuvant and patch test (APT) method of guinea pig sensitization testing was modified in 2 ways, s-APT and s-APT(2), in order to shorten the test period. These short-term test methods consist of 72-h closed application of test material with intradermal injection of emulsified Freund's complete adjuvant (E-FCA) for 1st induction, 48-h closed application of test material with (s-APT) or without (s-APT(2)) intradermal injection of E-FCA on the 7th day for 2nd induction, and open application on the 14th day for challenge. They were compared with conventional APT by using 8 allergenic chemicals (formaldehyde, nickel sulfate, cobalt sulfate, ethyl-p-aminobenzoate (benzocaine), isoeugenol, 2-mercaptobenzothiazole, 2,4-dinitrochlorobenzene (DNCB) and 1-phenylazo-2-naphthol (Sudan I)). The short-term methods gave similar results to those of conventional APT in terms of mean response, sensitization rate and sensitization potency (challenge concentration that induces a mean response equal to 1.0). Thus, our short-term methods, which are capable of evaluating skin sensitization within 17 days, are sufficiently sensitive to detect potentially hazardous contact allergens.

**Key words:** short-term guinea pig sensitization test; adjuvant and patch test (APT); s-APT; s-APT(2); formaldehyde; nickel sulfate; cobalt sulfate; ethyl-p-aminobenzoate (benzocaine); isoeugenol; 2-mercaptobenzothiazole; 2,4-dinitrochlorobenzene (DNCB); 1-phenylazo-2-naphthol (Sudan I). © Munksgaard, 2001.

*Accepted for publication 4 September 2000*

Skin sensitization tests using guinea pigs are generally employed to determine the risk of contact sensitivity of chemical compounds. Since Landsteiner et al. (1) first established a sensitization test method with guinea pigs, many skin sensitization tests have been developed and used for the evaluation of sensitizing potential. They can be divided into 2 groups, i.e., with or without the use of Freund's complete adjuvant (FCA). Among these test methods, both the guinea pig maximization test (GPMT) developed by Magnusson & Kligman (2) and the Buehler test (3) have been generally used worldwide for over 20 years. These methods are widely accepted by regulatory authorities and are listed in various guidelines, including one by the OECD (4). The GPMT is capable of predicting the skin sensitization potency of chemical compounds with high sensitivity and good reproducibility, but it has some limitations in that some compounds and materials are unsuitable for testing by the GPMT, because of their poor solubility in

water or other properties incompatible with the intradermal injection procedure.

Methods such as the adjuvant and patch test (APT) (5), split adjuvant technique (6) and cumulative contact enhancement test (CCET) (7) have been developed to overcome the above drawbacks, and have been used to evaluate the sensitization potency of insoluble materials. However, they require about 4 weeks until the sensitization potency can be judged, because an induction period of about 2 weeks is needed before the challenge test. Nevertheless, little work has been done to shorten the test period, except for one report about shortening the AP2 test by Kashima et al.(8).

We have reexamined the procedure of APT, in which test materials are applied percutaneously, and have developed new short-term methods with equivalent sensitivity to APT. In this paper, we present 2 short-term modified APT procedures for detecting contact sensitization, and we compare the results with those obtained in the con-

ventional APT method, using a panel of 8 contact allergens.

## Materials and Methods

### Animals

Hartley strain female albino guinea pigs, weighed 380 to 500 g at the beginning of the test, were purchased from Japan SLC Inc. The animals were housed individually in a stainless steel cage (260×380×180 mm) under controlled conditions (temperature, 23±3°C; relative humidity, 55±15%; lighting, a 12 h light and dark cycle). They were given a pelleted diet (RC-4, Oriental Yeast Co., Ltd., Japan) and sterilized water ad libitum. The present study was approved by the ethics committee of Shiseido Research Center in accordance with the guideline of National Institute of Health. 5 animals were distributed to both sensitizing and control groups with all chemicals.

### Chemicals

Cobalt sulfate and 2-mercaptobenzothiazole (2-MBT) were obtained from Aldrich Chemical Co., USA., nickel sulfate and ethyl-p-aminobenzoate (benzocaine) were from Sigma Chemical Co., USA., isoeugenol and 1-phenylazo-2-naphthol (Sudan I) were from Tokyo Kasei Kogyo Co., Ltd., Japan, 2,4-dinitrochlorobenzene (DNCB) was from Wako Pure Chemical Industries, Ltd., Japan and formaldehyde (36% w/w sol.) was from Taab Laboratories, UK. Sudan I was recrystallized from n-hexane and ethyl acetate before use, and other chemicals were used without purification.

The vehicles employed were purchased from the following producers: Otsuka Pharmaceuticals Co., Ltd., Japan (distilled water) and Wako Pure Chemical Industries, Ltd., Japan (ethanol and acetone). Freund's complete adjuvant (FCA) was purchased from Difco Laboratories, USA.

### Contact sensitization test

**Adjuvant and patch test (APT).** APT was performed as reported (5). On the 1st day, 0.1 ml of emulsified FCA (E-FCA), prepared from equal volumes of FCA and sterile distilled water, was injected intradermally at the 4 corners of a clipped and shaved 2×4 cm<sup>2</sup> nuchal area. A criss-cross lattice of abrasions was made at each site of injection. Aliquots of 0.1 ml of each test material were applied occlusively for 24 h on the injection sites of E-FCA. Similar abrasions and applications of test materials were repeated on the following 2 days. On the 8th day, the nuchal area was treated with 10% sodium lauryl sulfate in petrolatum. On the

9th day, 0.2 ml of each test material was again applied occlusively for 48 h. On the 21st day after initial induction, 0.01 ml aliquots of various concentrations of the test material in the vehicle were applied to the shaved area of the flank of each animal for challenge. The concentrations and vehicles used in both the induction and the challenge phase are shown in Table 1. Control animals were injected with E-FCA and treated occlusively with a vehicle in the same way as described for induction of the test groups, then on the 21st day, various concentrations of test material in the vehicle were applied to the shaved area of the flank of each animal for challenge.

**Shortened adjuvant and patch tests (s-APT and s-APT(2)).** Both s-APT and s-APT(2) were modified from APT (Fig. 1). On the 1st day, 0.1 ml of E-FCA was injected intradermally at the 4 corners of a shaved 2×4 cm<sup>2</sup> nuchal area. A criss-cross lattice of abrasions was made at each site of injection. Aliquots of 0.1 ml of test material were applied occlusively for 72 h on the injection sites of E-FCA. On the 7th day, 0.1 ml of E-FCA was injected intradermally at 2 sites in the same skin region only in the s-APT protocol, and 0.4 ml of material was again applied occlusively for 48 h in the nuchal area after making a criss-cross lattice of abrasions in both methods. On the 14th day after initial induction, challenge was performed in the

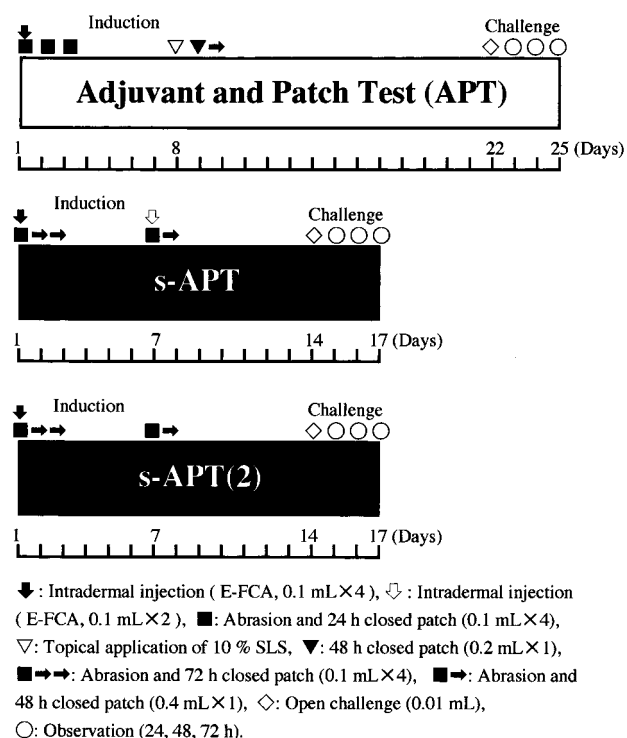


Fig. 1. Protocols of the sensitization test methods in guinea pigs.

Table 1. Chemicals examined in this study

| Chemical          | Source <sup>a)</sup> | Concentration |             | Vehicle <sup>b)</sup> |
|-------------------|----------------------|---------------|-------------|-----------------------|
|                   |                      | induction     | challenge   |                       |
| formaldehyde      | TAAB                 | 1%            | 0.1–5%      | DW                    |
| NiSO <sub>4</sub> | Sigma                | 3%            | 0.03–3%     | DW                    |
| CoSO <sub>4</sub> | Aldrich              | 3%            | 0.003–3%    | DW                    |
| benzocaine        | Sigma                | 10%           | 0.1–10%     | ethanol               |
| isoeugenol        | Tokyo Kasei          | 10%           | 0.1–10%     | ethanol               |
| 2-MBT             | Aldrich              | 1%            | 0.1–5%      | ethanol               |
| DNCB              | Wako                 | 1000 ppm      | 10–1000 ppm | acetone               |
| Sudan I           | Tokyo Kasei          | 10 ppm        | 1–1000 ppm  | acetone               |
|                   |                      | 30 ppm        | 1–1000 ppm  | acetone               |
|                   |                      | 100 ppm       | 1–1000 ppm  | acetone               |

<sup>a)</sup> TAAB Laboratories Equipment Ltd. (UK); Sigma Chemical Co. (St. Louis, USA); Aldrich Chemical Co. (Milwaukee, USA); Tokyo Kasei Kogyo Co. (Japan); Wako Pure Chemical Industries, Ltd. (Japan). <sup>b)</sup> DW: distilled water.

Table 2. Evaluation of skin reaction (scale for scoring)

|                                       |   |
|---------------------------------------|---|
| (1) Erythema formation                |   |
| no erythema                           | 0 |
| very slight erythema                  | 1 |
| well defined erythema                 | 2 |
| moderate to severe erythema           | 3 |
| severe erythema with eschar formation | 4 |
| (2) Edema formation                   |   |
| no edema                              | 0 |
| slight edema                          | 1 |
| moderate edema                        | 2 |
| severe edema                          | 3 |
| overall maximum score                 | 7 |

$$\text{sensitization rate (\%)} = \frac{\text{no. animals with positive reaction}}{\text{no. animals tested}} \times 100$$

$$\text{mean response} = \frac{\sum [(1) + (2)]}{\text{no. animals tested}}$$

same manner as in APT. The concentrations and vehicles used in both the induction and the challenge phase are shown in Table 1. Induction concentrations of chemicals were decided based on previous reports (8–10) except for isoeugenol and benzocaine which were decided according to unpublished in-house data.

#### Assessment of skin reaction

The skin reactions at the challenge sites were evaluated at 48 h after the challenge application, according to the criteria shown in Table 2. A score greater than 1 was considered as positive. The results were expressed as the mean score and frequency of positives. The summation of numerical scoring more than 1 was judged as a positive reaction because no skin reactions were elicited in control groups by any chemicals.

#### Histopathology

For histopathological examination, animals sensitized with 100 ppm Sudan I using either the APT or the s-APT(2) protocol were used. After observation of the skin reactions at 48 h, the animals were euthanized under deep anesthesia with diethyl ether and the skin at the challenge site and the cervical lymph nodes were removed. Tissue samples were fixed in 10% neutral buffered formalin solution and processed routinely for paraffin embedding, and sections were stained with hematoxylin and eosin (H&E).

#### Results

The results for mean response are shown in Fig. 2. In the cases of nickel sulfate and benzocaine, APT showed slightly higher mean response values than s-APT and s-APT(2). Also, in the case of cobalt sulfate, APT gave slightly higher mean response values than s-APT(2). On the other hand, in the animals exposed to formaldehyde, s-APT and s-APT(2) showed slightly higher mean response values than APT. Isoeugenol showed the highest mean response values in s-APT. In the case of DNCB, mean response values were almost the same in APT and s-APT(2). Among the other chemicals, Sudan I with 3 different induction concentrations and 2-MBT, there was no evident difference in mean response values among the 3 test methods examined.

The results for sensitization rate are shown in Fig. 3. In the cases of nickel sulfate and benzocaine, APT showed slightly higher sensitization rates than s-APT and s-APT(2). In the case of DNCB, s-APT(2) showed slightly higher sensitization rates than APT. On the other hand, in the case of formaldehyde, s-APT gave slightly higher sensitization rates than APT and s-APT(2). In the animals exposed to cobalt sulfate, the sensitization rates were almost the same in APT and s-APT(2). Among the other chemicals, Sudan I with 3 different induction concentrations, isoeugenol and 2-MBT, there was no evident difference in sensitization rates among the 3 test methods examined.

Sensitization potency of each chemical, expressed as the challenge concentration that induces a mean response equal to 1.0, in the 3 test methods is shown in Table 3. In the case of nickel sulfate, APT revealed 10× higher sensitivity than the 2 short-term methods. However, other chemicals gave similar results in all 3 test methods.

Histopathologically, acanthosis, parakeratosis and spongiosis of the epidermis and mononuclear cell infiltration of the dermis in the skin at the challenge site were observed at similar levels in the

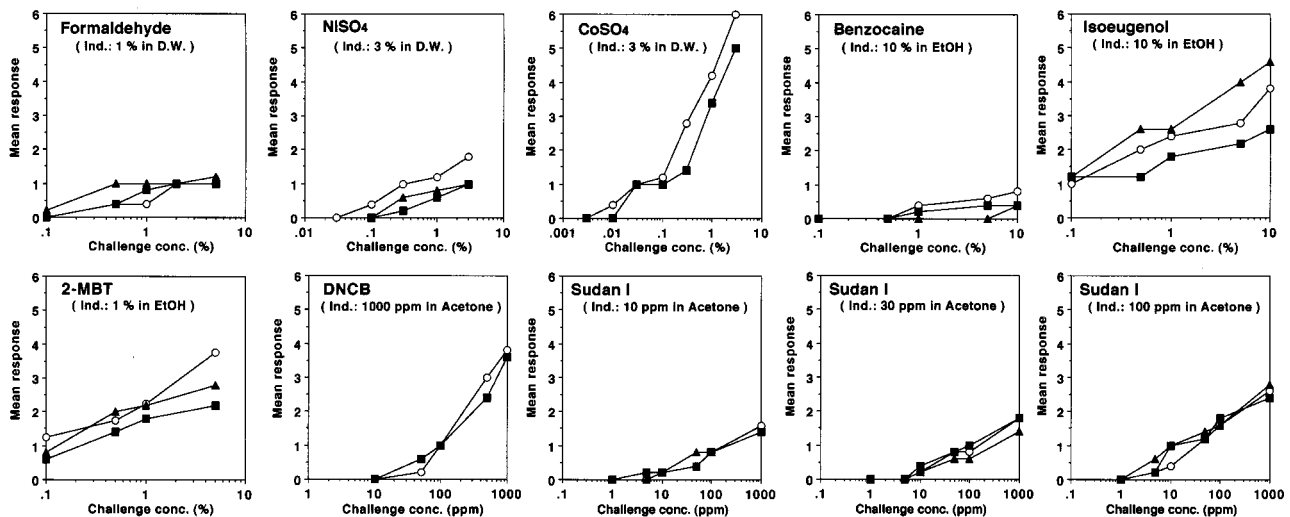


Fig. 2. Relationships between challenge concentration and mean response in the 3 methods. Animals were sensitized and then challenged with each chemical. Cobalt sulfate and DNCB were not examined by s-APT. The symbols  $\circ$ ,  $\blacktriangle$  and  $\blacksquare$  represent APT, s-APT and s-APT(2), respectively.

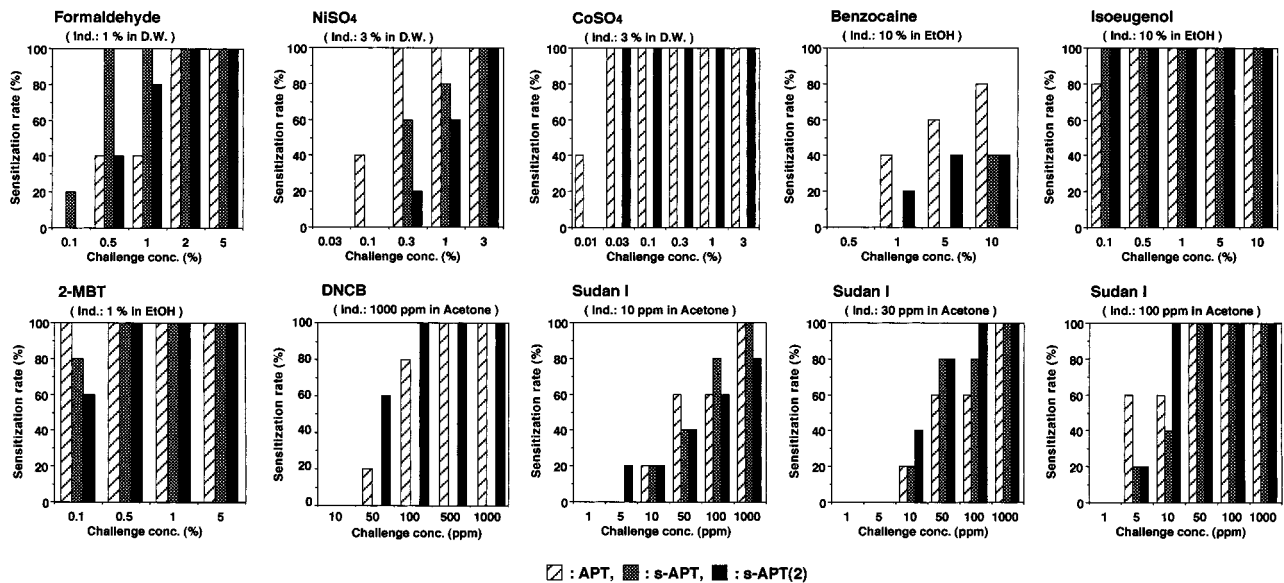


Fig. 3. Relationships between challenge concentration and sensitization rate in the 3 methods. Animals were sensitized and then challenged with each chemical. Cobalt sulfate and DNCB were not examined by s-APT.

animals that were sensitized with Sudan I in both the APT and s-APT(2) protocols (Fig. 4). Also, in the cervical lymph node, levels of follicular and paracortical hyperplasia in the cortex were equivalent in both methods (Fig. 5).

### Discussion

In this study, we compared 2 modified short-term skin sensitization tests, s-APT and s-APT(2), with the conventional APT using a panel of contact allergens, which included both water-soluble and lipid-soluble compounds. The s-APT and s-

APT(2) protocols both showed skin sensitization reactions similar in degree and frequency to those observed in the APT method. The test period needed for either s-APT or s-APT(2) was 8 days shorter than that for APT, but all 3 tests showed similar sensitivities for the detection of contact allergens. These results indicate that sufficient sensitivity was maintained even when the overall test duration was shortened to 17 days. When the skin at the challenge site and the cervical lymph nodes at the challenge phase were taken from the animals sensitized with 100 ppm Sudan I using the s-APT(2) and APT protocols, the histopathological

Table 3. Sensitization potency of chemicals

| Chemical          | Concentration |             | Sensitization potency <sup>a)</sup> |                 |              |
|-------------------|---------------|-------------|-------------------------------------|-----------------|--------------|
|                   | induction     | challenge   | APT                                 | s-APT           | s-APT(2)     |
| formaldehyde      | 1%            | 0.1–5%      | 2%                                  | 0.5%            | 2%           |
| NiSO <sub>4</sub> | 3%            | 0.03–3%     | 0.3%                                | 3%              | 3%           |
| CoSO <sub>4</sub> | 3%            | 0.003–3%    | 0.03%                               | – <sup>b)</sup> | 0.03%        |
| benzocaine        | 10%           | 0.1–10%     | 5%                                  | 5%              | 5%           |
| isoeugenol        | 10%           | 0.1–10%     | 0.1%                                | 0.1–0.5%        | <0.1%        |
| 2-MBT             | 1%            | 0.1–5%      | <0.1%                               | 0.1–0.5%        | 0.1–0.5%     |
| DNCB              | 1000 ppm      | 10–1000 ppm | 100 ppm                             | – <sup>b)</sup> | 100 ppm      |
| Sudan I           | 10 ppm        | 1–1000 ppm  | 10–100 ppm                          | 10 ppm          | 10 ppm       |
|                   | 30 ppm        | 1–1000 ppm  | 100–1000 ppm                        | 100–1000 ppm    | 100 ppm      |
|                   | 100 ppm       | 1–1000 ppm  | 100–1000 ppm                        | 100–1000 ppm    | 100–1000 ppm |

<sup>a)</sup> The challenge concentration that induces a mean response approximately equal to 1.0. <sup>b)</sup> Cobalt sulfate and DNCB were not examined by s-APT.

findings were essentially the same in both cases. Similar histopathological findings in the skin reaction sites were reported in humans with allergic contact dermatitis (11–13). Therefore, it is suggested that the mechanisms of the sensitization process and reactivity are quantitatively and qualitatively similar in all 3 methods.

Magnusson & Kligman (14) reported that a 2-week interval was needed after the last induction in order to achieve full sensitization in the GPMT. Most subsequent sensitization tests have also used an interval of about 2 weeks before challenge (5–

7,15–19). In other words, most researchers seemed to believe that 2 weeks are needed for completion of antigen-specific T cell proliferation at peripheral lymph nodes for the 2nd contact. In contrast, our results show that the s-APT and s-APT(2) protocols, which have an interval of 5 days between the induction and the challenge, can elicit a sufficient degree of sensitization. A similar result was reported by Kashima et al. (8) in the AP2 test, which is also a short-term method. They found that AP2, a modified method based on CCET, could detect most of the tested allergens more effectively than

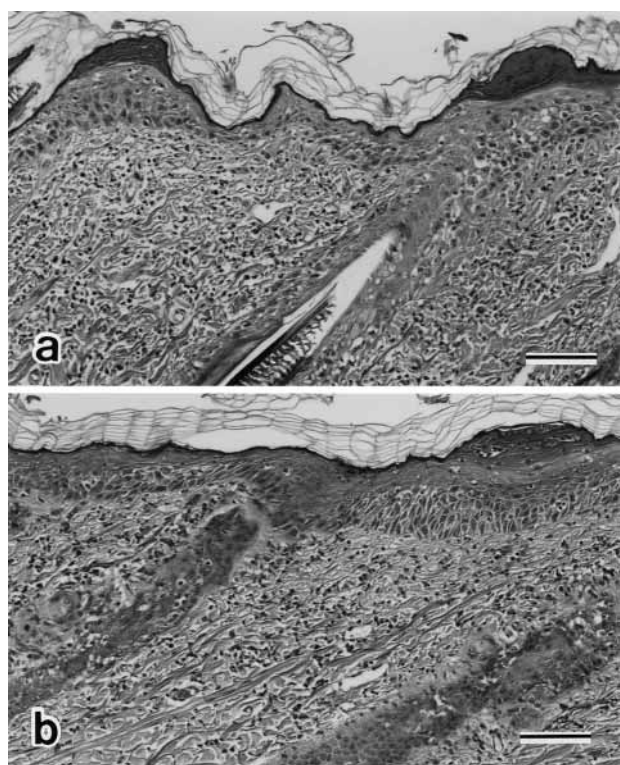


Fig. 4. Light micrographs of the skin at the challenge site from a guinea pig sensitized with Sudan I by the APT (a) and s-APT(2) (b) protocols. H&E stain. Bar=0.1 mm.

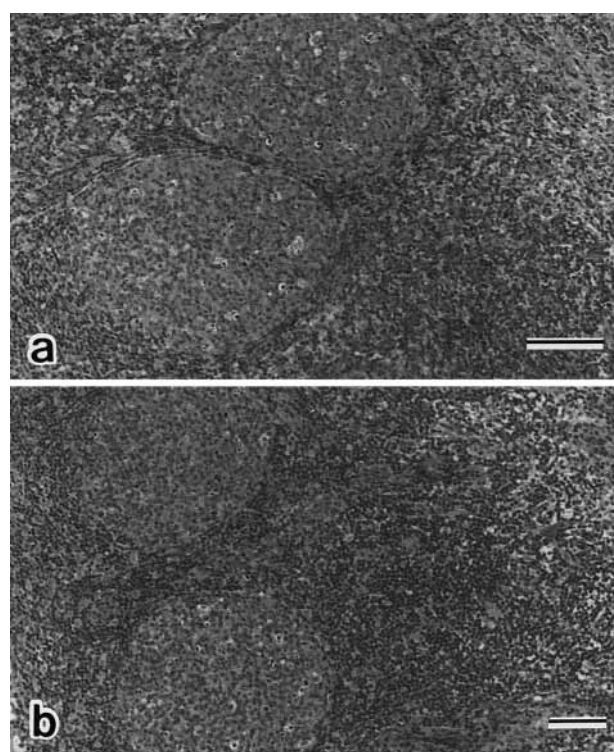


Fig. 5. Light micrographs of the cervical lymph node from a guinea pig sensitized with Sudan I by the APT (a) and s-APT(2) (b) protocols. H&E stain. Bar=0.1 mm.

CCET or GPMT. Our experiments support the idea that 5 days is long enough to sensitize guinea pigs under these experimental conditions. Thus, we confirmed that it is possible to shorten the test period without decreasing the detection sensitivity in the guinea pig sensitization test.

We conclude that s-APT and s-APT(2) are capable of evaluating skin sensitization potential within 17 days with sufficient sensitivity for the detection of potentially hazardous contact allergens. Furthermore, s-APT(2), which does not include the injection of E-FCA at 2nd induction, showed the same sensitivity as s-APT. Thus, we think that s-APT(2) without E-FCA for 2nd induction may be the preferred method from the viewpoints of simplicity and animal welfare. Further studies are needed to establish whether s-APT(2) will be useful not only for hazard identification, but also for risk assessment of contact allergens.

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