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# Immune complex induced vascular reactions in the mouse skin

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**A sensitive bioassay has been developed to study the mechanism of vascular reactions induced by immune complexes in the mouse skin. The antigen-antibody complex was prepared to known antigens, sheep red blood cells (SRBC) and bovine serum albumin (BSA), by incubating them with anti-SRBC and anti-BSA serum raised in BALB/c mice respectively. An aliquot (0·05ml) of each reaction mixture was injected intradermally at four sites of BALB/c mice. The inner surface of the skin was observed for neovascular responses. The sequence of reactions in the immune complex injected site were (*i*) reddish appearance and swelling at the injected site on day 2; (*ii*) yellowish nodule formation on day 5; (*iii*) appearance of new blood vessels around the nodule on day 8; and (*iv*) complete regression of pathological reaction by day 15. Histological examination revealed the presence of a thick layer of polymorphonuclear leukocytes (PMN) around the injected cells and numerous new blood vessels surrounding the PMN nodule. Cytocentrifuged smear preparations showed that on the day 5, the nodule consisted of more than 95 per cent PMN. The findings demonstrate that this skin test could be developed into a sensitive bioassay to determine the functional significance of the soluble immune complexes in clinical situations. Further, the assay may be a useful model to analyse the mechanism of immune complex induced vascular pathology and to identify the various cellular and humoral components associated with this phenomenon.**

The importance of neovascularization is well recognised in a variety of disease processes, in inflammatory reactions, in delayed hypersensitivity responses and more specifically as an essential component of tumour growth[1-4]. Localized vascular reaction, moreover, plays a major role in the pathogenesis of several immune complex-mediated diseases like rheumatoid arthritis, systemic lupus erythematosus and acute glomerulonephritis[5,6]. Further, the role of immune complexes in the pathogenesis of vascular lesions has been demonstrated in experimental models[7]. However, our understanding of the mechanism underlying such induced neovascularization in diseases as well as the various contributing components associated with this phenomenon is still limited.

For the estimation of soluble circulating immune complexes (CICs) in the peripheral blood, over 20 techniques, both qualitative and quantitative have been

employed[8]. These methods are designed on the basis of size, surface property, solubility, electrical charges and ohter biological properties of soluble immune complexes. However, they lack the required specificity and moreover, the relationship between the level of CICs and the clinical symptom is not fully understood[8]. By means of an *in vitro* (Boydon Chamber technique) neutrophil migration assay, sera from rheumatoid arthritis patients containing CICs was shown to inhibit neutrophil migration[9]. Thus the possibility of developing this assay for the detection of CICs was indicated. However, the authors could not find significant correlation between rheumatoid factor titres and the inhibition of neutrophil migration. Therefore, the present study was undertaken to develop a bioassay in the mouse skin to understand the mechanism of vascular reactions induced by immune complex.

## Material & Methods

*Animals* : Inbred strain of BALB/c (6 to 8 wk old male) mice from our own colony were used.

*Antigens and antisera* : Sheep red blood cells (SRBC) and bovine serum albumin (BSA, Sigma) were used as antigens. Required concentration of antigens was prepared before use. Two groups of mice were immunized, one with SRBC and the other with BSA following the standard protocol to raise high titre antisera[10,11]. Animals were bled, sera collected, pooled and inactivated at 56°C for 30 min, stored at —20°C and used within three weeks. Anti-SRBC antiserum was titrated to determine the subagglutinating level of antibody activity. For the titration of anti-BSA antiserum, passive haemagglutination assay test was performed, using SRBC coated with BSA[12].

*Antigen-antibody complex* : Washed SRBC suspension (40%) was mixed with equal volume of antisera diluted to its subagglutinating level and the mixture incubated at 37°C for 1 h and then for 4 h at 4°C before injection. Similarly, BSA coated-SRBC was treated with anti-BSA antiserum. In order to localize the immune complex at the injected site, the complex was presented in particulate form. Fresh normal mouse serum (heat inactivated at 56°C for 30 min), incubated with SRBC was used as control. Anti-SRBC antiserum along with rat red blood cells and anti-BSA antiserum along with SRBC were used as additional controls.

*Skin assay* : Intradermal injections were performed with a 26-gauge needle, following the technique of Sidky and Auerbach[13]. An aliquot of 0·05 ml of reaction mixture (SRBC-anti-SRBC antiserum, BSA-anti-BSA antiserum) was injected intradermally at two sites in the right flank. Similarly, control preparation was injected at two sites in the left flank of the same animal.

In order to follow the vascular reactions, mice were killed by anaesthetic ether, a mid-ventral incision was made in the skin and inner surface of the skin was observed under a dissecting microscope with the help of transmitted light. The site of injection was exposed by carefully removing fatty tissue covering the area and it was also required to stretch the skin gently with forceps to observe the newly developed blood vessels.

*Histology* : Histological preparation of the injected site of the skin was made from Bouin's fixed material using paraffin

embedding and haematoxylin eosin staining. Cytocentrifuged smear was stained with May-Grunwald Giemsa to identify the cell types present in the nodule.

## Results

*Gross observation of the injected site* : 2 to 12 days after the injection of immune complex or control serum, inner surface of the skin was examined for neovascular responses. Data from 140 mice (560 sites of injection) are presented in Table I for SRBC-anti-SRBC antiserum and Table II for BSA-anti-BSA antiserum. In the controls, the injected site appeared reddish and flat up to 8 days and then brownish by day 10. Subsequently, the injected area appeared normal. None of the controls showed nodule formation or neovascularization.

In the case of SRBC-anti SRBC antiserum injected sites, initially the injected area appeared reddish with slight swelling by day 3. At each injected site a yellowish nodule was prominent by day 5 and new blood vessels were detected by day 8 in more than 90 per cent of the cases (Table I). Haemorrhage was also observed around the nodule on day 6. Interesting feature of this neovascularization is that new capillaries were clearly directed towards the nodule from the host vessels (Fig. 1). By about day 10, injected site became brownish and flat, and subsequently regained the normal appearance.

The same sequence of reactions were observed in BSA-anti-BSA antiserum injected site (Table II). In controls (BSA coated SRBC with normal mouse serum or uncoated cells treated with anti-BSA antiserum), neither nodule formation nor neovascularization was observed.

**Table I.** SRBC-anti-SRBC serum induced vascular reactions in the skin of BALB/c mice

| Day* of observation | Type of reaction | No. of positive/total cases | |
|---|---|---|---|
| | | Experiment** | Control† |
| 2 | Reddish | 28/30 | 26/30 |
| 3 | Swelling | 30/30 | 0/30 |
| 5 | Yellowish nodule | 29/32 | 0/32 |
| 6 | Haemorrhage around the nodule | 10/20 | 0/20 |
| 8 | New blood vessels | 29/32 | 0/32 |
| 10 | Brownish, flat | 32/32 | 32/32 |

*Day on which the animals were killed and injected sites observed. **Intradermal injection of SRBC-anti-SRBC antiserum (used within 2 wk after bleeding. †Intradermal injection of SRBC-with heat inactivated normal mouse serum

**Table II.** BSA-anti-BSA serum induced vascular reactions in the skin of BALB/c mice

| Day of observation* | Type of reaction | No. of pegitive/total cases | |
|---|---|---|---|
| | | Experimental† | Control‡ |
| 2 | Reddish | 24/24 | 12/24 |
| 3 | Swelling | 24/24 | 0/24 |
| 5 | Yellowish nodule | 20/20 | 0/20 |
| 6 | New blood vessels | 20/20 | 0/20 |
| 10 | Brownish, flat | 16/16 | 16/16 |

*Day on which the animals were killed and injected site observed. †Intradermal injection of BSA-anti-BSA antiserum (used within 2 wk after bleeding. ‡Intradermal injection of either BSA-coated cells with normal mouse serum or uncoated cells with anti-BSA

The sequence of vascular reactions was also followed histologically. During the second day, injected cells were seen packed within the dermal tissue in both control and experiment. From third day onwards a large number of PMN was seen in the immune complex injected site. Most of the veins at the injected site were surrounded by PMN, indicating the profound migration of PMN towards the site of injection of antigen-antibody complex (Fig. 2). This was followed by the formation of a nodule, consisting of a central core of injected cells surrounded by a thick layer (about 2 mm) of PMN (Fig. 3). By sixth day, PMN were rarely seen around the blood vessels (Fig. 4) indicating the absence of any further influx of PMN into the injected site. At this point, numerous new blood vessels (Fig. 4) had developed around the nodule and the PMN layer had started reducing in size. The clearance process of injected cells was completed by tenth day. In controls neither PMN infiltration nor formation of new blood vessels was seen at the injected site (Fig. 5).

Cells were isolated from the nodules and enumerated. A fifth day nodule consisted of 5 to $10 \times 10^6$ cells. Cytocentrifuged smear preparation showed that the nodule consisted of mainly (>95%) PMN, the remaining cells being macrophages (Fig. 6).

## Discussion

Observations by Izhizaka and Campbell[14] showed that antigen-antibody complexes injected into skin produced a marked local increase in capillary permeability. Arthus-reactions are occasionally observed in modern clinical practice when patients are subjected to skin test with various antigens. Thus, there is large clinical and experimental evidence to suggest that the skin may be an organ which by its very physical properties and distribution of blood supply, is susceptible to microvascular injury induced by immune complexes.

In the present study, we have developed a reproducible and simple technique to understand the mechanism of neovascularization induced by immune complexes in the mouse skin. The importance of PMN in this process has also been demonstrated. Histological examination showed that within a short period after the injection of antigen-antibody complexes, PMN marginate and emigrate through vessel walls into surrounding tissue spaces and then migrate towards the site of injection of antigen-antibody complex. The PMN infiltration and the processing of immune complexes by PMN have been well demonstrated in experimental animal models[15]. Further, the PMN infiltration towards the injected site leads to the formation of new blood vessels as already described in the rabbit corneal model.[16]

As revealed by our earlier studies[17], it was possible to collect a high number of activated PMN from the site of injection of the immune complex in the skin. When these cells were injected into the skin of different groups of mice, they were able to induce inflammatory response within 48h (K. Sailendri, Unpublished data).

It is proposed to carry out further studies using mouse skin model, on the nature of new vessels induced by immune complexes, and factors responsible for the vascular reaction and also to identify the natural nhibitors of angiogenesis.

The phenomenon demonstrated in the mouse skin resembles 'Arthus reaction'

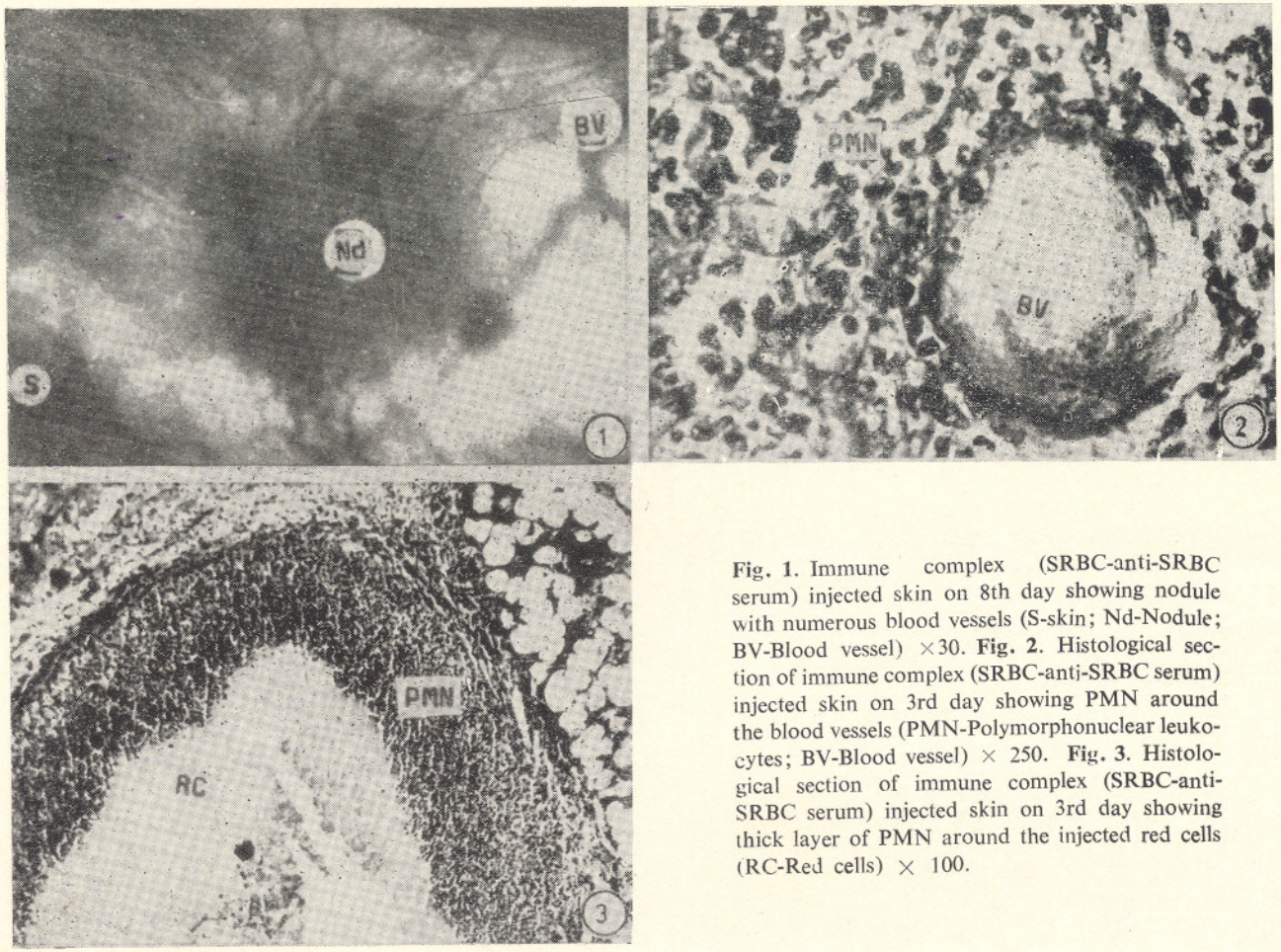
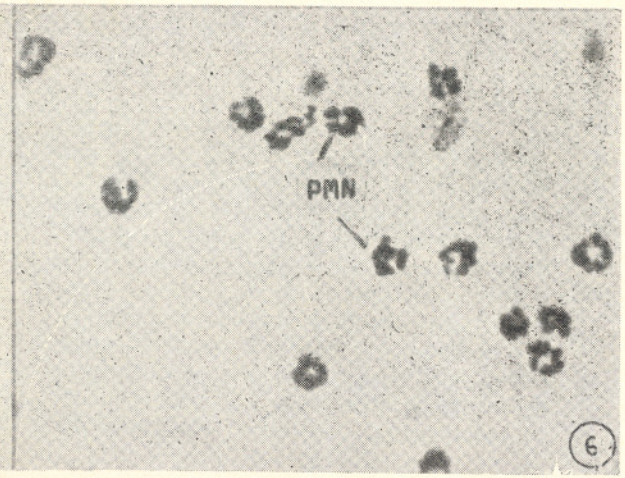



**Fig. 1.** Immune complex (SRBC-anti-SRBC serum) injected skin on 8th day showing nodule with numerous blood vessels (S-skin; Nd-Nodule; BV-Blood vessel) ×30. **Fig. 2.** Histological section of immune complex (SRBC-anti-SRBC serum) injected skin on 3rd day showing PMN around the blood vessels (PMN-Polymorphonuclear leukocytes; BV-Blood vessel) × 250. **Fig. 3.** Histological section of immune complex (SRBC-anti-SRBC serum) injected skin on 3rd day showing thick layer of PMN around the injected red cells (RC-Red cells) × 100.

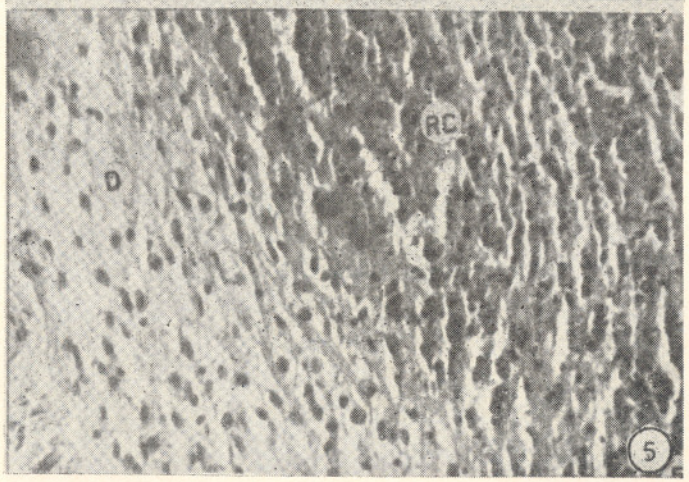




**Fig. 4**. Histological section of immune complex (SRBC-anti-SRBC serum) injected skin on 6th day showing invasion of PMN inside the injected area and clearance of PMN around the blood vessels × 100. **Fig. 5**. Histological section of SRBC-NMS (normal mouse serum) injected skin on 3rd day showing injected red cells alone without PMN infiltration (RC-Red cells; D-Dermis) × 100. **Fig. 6**. Cytocentrifuged smear preparation of immune complex injected portion on 5th day showing PMN and few macrophages (M-Macrophage) × 250.

in many respects. Hence, the skin assay provides two important advantages (i) it can be used as a bioassay to determine the functional potential of immune complex isolated from patients; and (ii) it would be possible to understand the mechanism of immune complex mediated hypersensitivity.

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