Effect of Primary Injection Site upon Cellular and Antitoxin Responses to Subsequent Challenging Injection*† (33413)

M. X. TURNER[1], R. S. SPEIRS, AND J. A. MCLAUGHLIN[2]

Quantitative techniques for the study of inflammatory cells which accumulate after an intraperitoneal injection of antigen were developed in our laboratory (1, 2). Results obtained not only confirmed a sequential cell response, but demonstrated quantitative differences in the pattern of reactions depending upon whether animals had been previously sensitized. Although the site of primary antigen injection has been shown to affect humoral and cellular antibody (3–5), it has not been demonstrated to affect the local inflammatory responses to subsequent injections of the same antigen. Since the site of primary injection can influence the immune response, it was considered of interest to apply quantitative procedures to determine whether the site of priming also influences the inflammatory response to subsequent injections.

Materials and Methods. The animals used in these experiments were female BDF_1 hybrid mice between 8 and 12 weeks of age at the time of priming. The antigen used was either fluid tetanus toxoid (FTT, Lederle) or alum precipitated tetanus toxoid (APTT, Lederle). Previous experiments had indicated that an intensive local cellular response and a high degree of immunization were induced when the animals were injected with fluid tetanus toxoid plus an adjuvant of pertussis vaccine (6). In the present experiments the animals were primed by an initial injection of 0.2 ml of FTT added to 0.2 ml of pertussis vaccine (diluted 1:10, Parke Davis Co.), injected either subcutaneously (s.q.) or intraperitoneally (i.p.). In all cases the final injection (0.2 ml of APTT) was administered i.p.

In group 1, the animals were primed s.q. and challenged i.p. 4 weeks later. In group 2 the animals were primed i.p. and challenged i.p. 4 weeks later. The animals in groups 3–6 received an additional sensitizing injection making a total of 3 injections. Group 3 was primed s.q. and 4 weeks later received a second s.q. injection (0.2 ml of APTT) with a subsequent challenge 2 weeks later (s.q.–s.q.–i.p.). Group 4 was treated in a similar manner except that the second injection was given i.p. (s.q.–i.p.–i.p.). Group 5 received an i.p. injection followed by a s.q. injection with an i.p. challenge (i.p.–s.q.–i.p.). Group 6 received all 3 injections i.p. (i.p.–i.p.–i.p.).

Five animals per point were autopsied 0, 1, 3, 5, 7, and 10 days after the last injection. The animals were killed by cervical dislocation, an incision was made in the abdomen, and a small aliquot of peritoneal fluid was removed, placed on a slide and smears were made using a fine artists brush (size 0) (7). The peritoneal contents were then washed into collecting tubes with 20 ml of cold Carpentier's solution (2). Estimates of the total number of peritoneal cells were made using a hemocytometer. The dried smears were fixed in methyl alcohol, stained in May-Grüenwald Giemsa, and at least 1000 cells/animal were counted to determine the proportion of each cell type. Mast cells were not included in the graphs since they were few in number and no marked differences were observed between groups.

In each group, 10 additional animals were bled via the tail vein at weekly intervals for determination of the amount of circulating antitoxin by biological assay in mice (8).

Results. The inflammatory responses and subsequent antitoxin titers to a challenging injection of tetanus toxoid were found to vary depending upon the site of the priming in-

* These investigations were supported in part by a grant from the Atomic Energy Commission AT(30-1)3024 to Dr. R. S. Speirs.

† Submitted by M. X. Turner in partial fulfillment of the requirements for the Ph.D. in the Department of Biological Science, Fordham University, Bronx, New York.

[1] Present address: Postdoctoral Fellow at Downstate Medical Center.

[2] Present address: Fordham University, Bronx, New York.

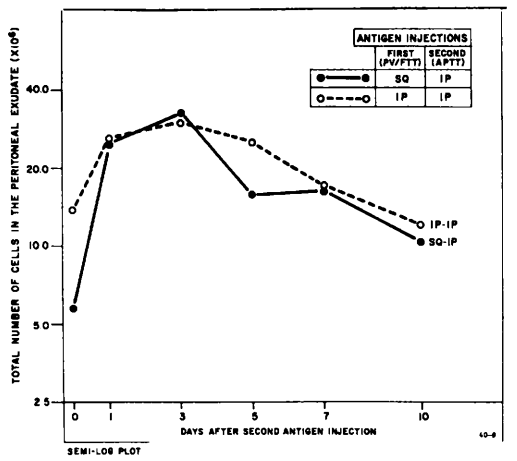

FIG. 1. The total number of cells responding to a challenging i.p. injection of antigen administered 4 weeks after priming. Except for days 0 and 5 there were no significant differences between group 1 (s.q.–i.p.) and group 2 (i.p.–i.p.). An increased number of cells at 0 day were present in group 2 which had received the priming injection intraperitoneally 4 weeks earlier (5 animals/point).

jection. Results obtained from each group in the experiment are presented separately.

In group 1, animals primed by a single s.q. injection, the total population of inflammatory cells in the peritoneal cavity was slightly more than 6 million at the time of challenge (Fig. 1, day 0). The i.p. challenge induced an inflammatory response, increasing the cellular population in the peritoneal cavity to 25 million within 1 day. A peak of 32 million occurred on day 3 followed by a decrease to approximately 10 million cells by day 10. The number and proportion of each cell type present in the inflammatory exudate is shown in Fig. 2. Prior to challenge 6 million mononuclear cells were present constituting 96% of the total population. One day after challenge, they rose to 13 million, peaking at 18 million on day 3. Neutrophils and eosinophils also migrated into the peritoneal cavity and constituted a high proportion of the resulting exudate population. These changes, while increasing the total cell population, resulted in a decrease in the proportion of mononuclear cells to 52% within 24 hr. The number of neutrophils in the peritoneal exudate was less then 70,000 (<2%) prior to challenge. They rose to a peak of 6 million (24%) on day 1, decreasing rapidly thereafter. Eosinophils numbered less than 100,000 (<2%) cells prior to challenge, but by day 1 they increased to 6 million (24%). The peak response of approximately 13 million (42%) on day 3 was followed by a decline to less than 2 million cells (12%) by day 10.

The total cell population of the peritoneal exudate in the group 2 animals 4 weeks after i.p. priming was more than 13 million (Fig. 1, day 0). The number of cells rose to a total of 26 million 1 day after challenge and reached a peak of 30 million on day 3, with a decrease to 12 million observed by day 10. Prior to challenge approximately 13 million mononuclear cells were present constituting 95% of the total population (Fig. 2, day 0). One day after challenge they increased to 19 million (71%) with a peak of 25 million (85%) on day 3. The neutrophils prior to challenge were just under 0.6 million (4%). One day after challenge, they increased to 6 million (23%) after which a slow decrease was noted until by day 10 they made up less

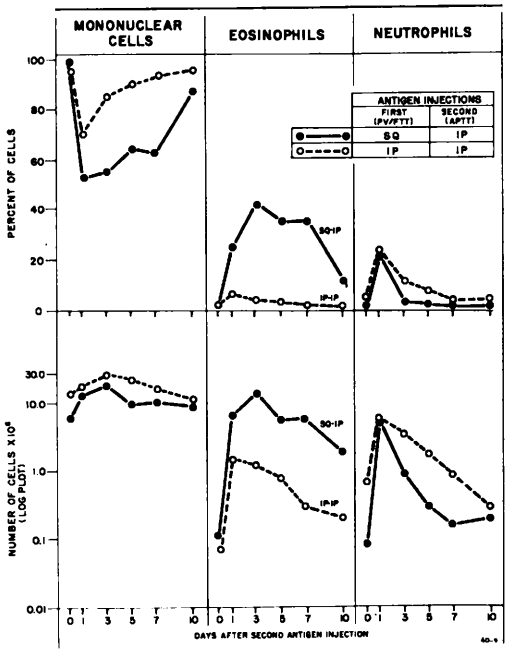

FIG. 2. The proportion (upper chart) and quantitative response (lower chart) of each cell type responding in groups 1 (s.q.–i.p.) and 2 (i.p.–i.p.) to a challenging injection 4 weeks after priming (5 mice/point).

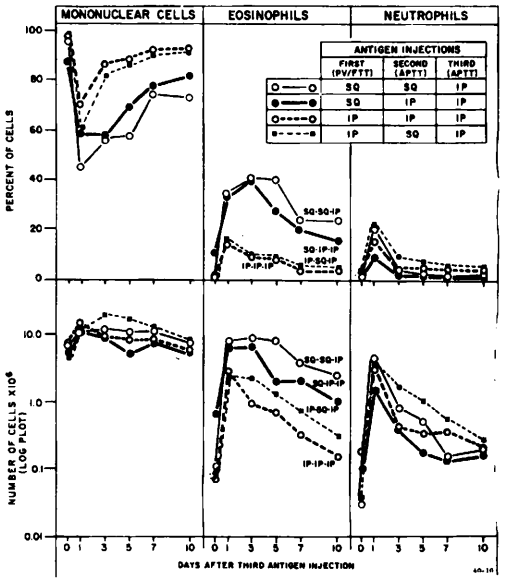

FIG. 3. The proportion (upper chart) and quantitative response (lower chart) of each cell type responding to a challenging injection (groups 3–6). The animals were immunized by 2 injections of tetanus toxoid given 2 and 6 weeks earlier (5 mice/point).

than 4% of the population. There were 80,000 (1%) eosinophils prior to challenge. They reached a peak of 1.5 million (6%) 1 day after challenge, and slowly declined in number thereafter.

In groups 3–6 the animals received a series of two sensitizing injections either s.q. or i.p. 4 weeks apart, and then were challenged i.p. 2 weeks after the second injection (Fig. 3). In all four groups, it was noted that the neutrophils rapidly increased in number peaking at 24 hr after challenge. The mononuclear cells likewise increased in number, usually peaking during the first 3 days. On the other hand, the eosinophil responses showed considerable variation between the different groups both in the number of cells taking part in the reaction and in the time of peak response.

In group 3 (s.q.–s.q.–i.p.) the number of eosinophils in the peritoneal cavity prior to injection was less than 60,000 (1%). They rose to 8 million (35%) 1 day after challenge, peaking at 9 million (43%) on day 3, and gradually decreased to 2 million (24%) by day 10.

In group 4 (s.q.–i.p.–i.p.) the eosinophils in the peritoneal exudate prior to challenge numbered approximately 0.6 million (10%). On the day after injection the cells rose to 6 million (34%), reaching a peak of 6.5 million (40%) on day 3 with a decrease to 1 million (15%) by day 10.

In group 5 (i.p.–s.q.–i.p.) the eosinophils rose from 70,000 (<2%) on 0 day to 2.5 million (15%) on day 1, then decreased thereafter.

In group 6 (i.p.–i.p.–i.p.) the eosinophils rose from 60,000 (1%) on 0 day to peak of 3 million (13%) on day 1 then decreased thereafter so that less than 200,000 (3%) were present on day 10.

The eosinophil responses obtained on the third day in each of the 6 groups is shown in Table I. Statistical comparisons were made and the significant differences noted.

The antitoxin responses are shown in Fig. 4. Following a single s.q. injection of antigen (col. 1 and 2), there were consistently fewer than 1000 antitoxin units/ml of blood during the 4-week period following priming. When the second injection was administered either s.q. (col. 1) or i.p. (col. 2), the titer rose to approximately 65,000 antitoxin units by 6 weeks. After the third injection administered i.p. (col. 1 and 2), the titer rose to 360,000 units at the end of 8 weeks.

When the primary injection was given i.p. (col. 3 and 4), the titer rose to 700 antitoxin units between the third and fourth week after injection. After the second injection administered s.q. (col. 3), the titer rose to 72,000 units within 2 weeks (week 6). A third injection given i.p. (column 3) resulted in an increase to 450,000 antitoxin units by the end of week 8.

In the last group (col. 4), the second i.p. injection caused an increase to 180,000 units within 2 weeks after challenge (week 6). When the third injection was administered i.p., the titer rose to more than 1,000,000 units 2 weeks later.

Discussion. Earlier reports had indicated differences in the cellular responses to antigen injections depending upon the degree of sensitization of the animals (9–11). It was noted that a priming injection of antigen in-

TABLE I. A Comparison of Eosinophil Responses on the Third Day after Challenge.

Group	Injection sites 1	2	3	Total no. of eosinophils (mean ± SEM; × 10^6)	Differences (groups compared)	Significance of difference*
1	s.q.	i.p.	—	13.26 ± 0.986	1 and 2	.01
					1 and 3	.05
					1 and 4	.01
2	i.p.	i.p.	—	1.18 ± 0.179	2 and 5	.05
					2 and 6	NS
3	s.q.	s.q.	i.p.	9.12 ± 0.930	3 and 4	.05
					3 and 5	.01
					3 and 6	.01
4	s.q.	i.p.	i.p.	6.53 ± 0.270	4 and 5	.01
					4 and 6	.01
5	i.p.	s.q.	i.p.	2.14 ± 0.351	5 and 6	.05
6	i.p.	i.p.	i.p.	0.94 ± 0.139		

* t test = .05 significant; .01 highly significant.

duced a local response resulting in the accumulation of millions of cells at the site of injection. Subsequent challenging injections induce a similar influx of mononuclear cells and neutrophils, and marked quantitative increases in the number of eosinophils.

Our observations indicate that there are differences in the cellular responses to a challenging i.p. injection depending upon the site of the priming injection. Differences are reflected in the time of peak response and in the number of eosinophils taking part in the inflammatory reactions. Animals challenged at 4 weeks after i.p. priming (group 2) had

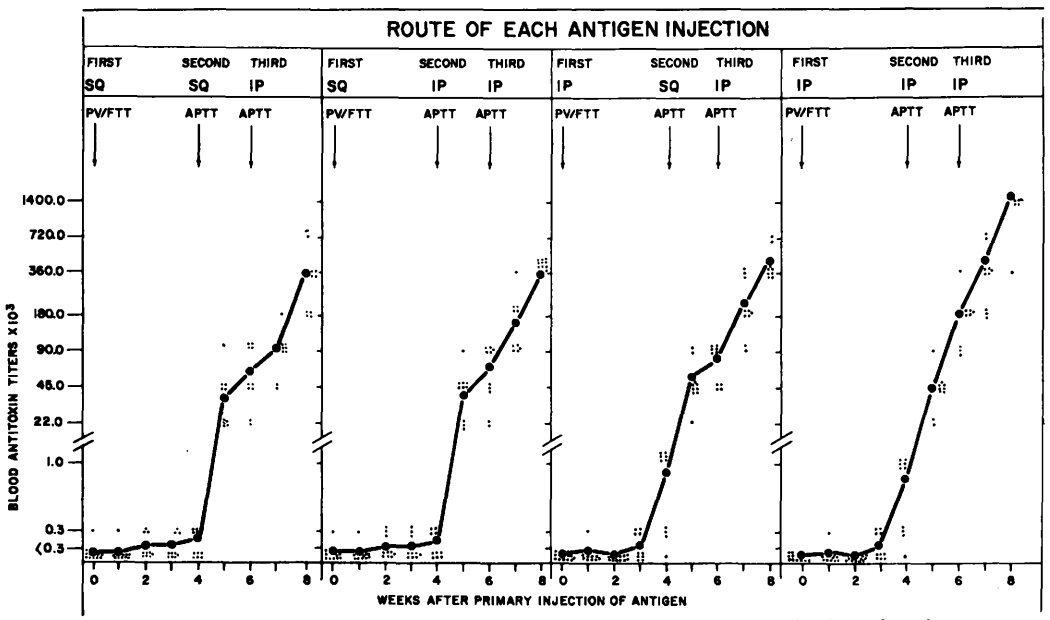

FIG. 4. The antitoxin titers measured at weekly intervals following each injection of antigen was determined by the toxin neutralization method. Each point represents a single animal; the line connects the average titer of 10 animals.

relatively low numbers of eosinophils, peaking at 24 hr. On the other hand, the number of eosinophils in the inflammatory exudate of s.q. (group 1) primed animals was significantly higher, peaking about 3 days after the challenging injection.

A variation in the local eosinophil response was also noted after the third injection. Animals receiving repeated i.p. injections (group 6) had fewer eosinophils in the inflammatory exudate on days 3–10 than did the animals receiving one or more subcutaneous sensitizing injections. On the other hand, the site of the sensitizing injection did not appear to significantly alter the neutrophil and mononuclear cell responses to subsequent challenging injections.

All animals, regardless of the site of the priming injection, showed an increase in antitoxin titers following the second injection. Animals receiving 2 i.p. injections (group 2) had consistently higher titers than those receiving subcutaneous injections or a combination of subcutaneous and intraperitoneal injections. When the antigen was injected for the third time, higher levels of circulating antitoxin were present, the highest titers being found in animals repeatedly injected i.p. (group 6).

The greatest accumulation of eosinophils occurred in challenged animals which had been primed earlier with a single s.q. injection (Table I). These animals had relatively low antitoxin titers at the time of challenge. The accumulation of eosinophils in animals given 2 subcutaneous injections prior to challenge (group 3) was significantly less than in those animals primed with only a single s.q. injection. On the other hand, animals receiving repeated i.p. injections had the lowest number of eosinophils in the exudate, and the highest antitoxin titers, both at the time of challenge and 10 days later. This latter group had circulating antitoxin titers that were more than 100 times higher than the s.q. primed animals at the time of challenge.

It has been repeatedly noted that a local eosinophilia occurs in chronic inflammation, (12, 13) especially in allergic patients (14), in parasitic infestations (15) or following anaphylactic reactions (16). A general assumption has been that the eosinophilia is caused by reaction of antigen with antibody (17–19). If antigen–antibody reactions per se were responsible for the accumulation of eosinophils, then it would be expected that animals with the highest circulating antitoxin titers at the time of challenge would exhibit the greatest number of eosinophils. However in our experiments the animals with the highest antitoxin titers had the least accumulation of eosinophils. Our data, therefore, does not lend support to the concept that antigen–antibody complexes per se induce the local eosinophil response. These data confirm results of earlier experiments in which passively immunized animals were shown to have no significant increase in eosinophils in response to an injection of specific antigen when compared with the control nonimmunized animals (20). In separate experiments utilizing immunized animals, passive transfer of antitoxin prior to challenge resulted in an inhibition of the eosinophil response. (Turner and Speirs, unpublished.)

The factors involved in local eosinophilia following antigen challenge, have been recently reviewed by Hudson (19). Histamine response (21), fibrin formation (22), hypersensitive cell reactions (23), and antigen–antibody complexes (18) have all been considered as eosinophil inducing factors. Since the present series of experiments suggest that eosinophils are not attracted to antigen–antibody complexes per se, further investigations are currently in progress.

Summary. The data presented indicates that the site of priming affects the local inflammatory cellular response to subsequent challenging injections. Animals sensitized with one or two subcutaneous injections showed a marked and persistent eosinophilia following a challenging intraperitoneal injection. On the other hand, animals sensitized by 1 or 2 intraperitoneal injections had consistently fewer eosinophils and they routinely peaked on day 1. Animals receiving 3 injections in which the site of the second injection differed from that of the primary had intermediate numbers of eosinophils. Neutrophils and mononuclear cells showed no consistent differences in their response pattern. Repeated i.p. injec-

tions produced higher antitoxin titers than any other combination tested. There appears to be an inverse correlation between the circulating antitoxin at the time of challenge and the subsequent eosinophil responses. Animals with high antitoxin titers have fewer eosinophils in the inflammatory exudate. These results do not support the concept that the local eosinophilia is due to antigen–antibody reactions per se, but suggest instead that the local eosinophilia may be inhibited by the presence of antibody at the time of challenge.

1. Dreisbach, M. E., Snell, G. E., and Speirs, R. S., J. Natl. Cancer Inst. 17, 297 (1956).

2. Speirs, R. S., Jansen, V., Speirs, E. E., Osada, Y., and Dienes, L., *in* "Tritium in the Physical and Biological Sciences," Vol. 2, p. 301, Intern. A.E.C., Vienna (1962).

3. Leskowitz, S. and Waksman, B. H., J. Immunol. 84, 58 (1960).

4. Stavitsky, A. B., *in* "Immunopathology of Uveitis" (A. R. Silverstein, ed.), p. 64. Williams and Wilkins, Baltimore, Maryland (1964).,

5. White, R. G., *in* "Mechanisms of Antibody Formation," p. 25. Academic Press, New York (1960).

6. Geller, B. D. and Speirs, R. S., Immunology 15, 707 (1968).

7. Burke, W. T., Brotherston, G., and Harris, C., Am. J. Clin. Pathol. 25, 1226 (1955).

8. Ipsen, J., J. Immunol. 72, 243 (1953).

9. Litt, M., Blood 16, 1318 (1960).

10. Speirs, R. S., Ann. N. Y. Acad. Sci. 59, 706 (1955).

11. Speirs, R. S., Blood 22, 363 (1963).

12. Ringoen, A. R., *in* "Handbook of Hematology" (H. Downey, ed.), Vol. 6, p. 179. Harper (Hoeber), New York (1938).

13. Riis, P., "The Cytology of the Inflammatory Exudate," p. 1. Munksgaard, Copenhagen (1959).

14. Campbell, A. C., Drennan, A. M., and Retthe, T., J. Pathol. Bacteriol. 40, 537 (1935).

15. Opie, E., Am. J. Med. Sci. 127, 477 (1904).

16. Campbell, D. H., J. Infect. Diseases 72, 42 (1942).

17. Litt, M., Blood 16, 1330 (1960).

18. Litt, M., Immunology 87, 521 (1961).

19. Hudson, G., Seminars Hematol. 5, 166 (1968).

20. Speirs, R. S., Res. Bull. 3, 19 (1957).

21. Archer, R. K., St. Johns Hosp. Dermatol. Soc. 50, 112 (1964).

22. Riddle, J. M. and Barnhart, M. I., Blood 25, 776 (1965).

23. Speirs, R. S., *in* "Tritium in the Physical and Biological Sciences," Vol. 2, p. 419. Intern. A.E.C., Vienna (1962).

Received July 10, 1968. P.S.E.B.M., 1968, Vol. 129.

The Effects of Thiocyanate on Mitochondrial Respiration* (33414)

GEORGE W. KIDDER III (Introduced by W. S. Rehm)

Biology Department, Wesleyan University, Middletown, Connecticut 06457

The thiocyanate ion, SCN^-, has been extensively employed as a reversible inhibitor of gastric acid secretion. We have reported cytochrome redox changes associated with SCN inhibition in bullfrog gastric mucosa (1) and, since we found no SCN^- inhibition of oxygen consumption in tissue mince, concluded that the primary action of SCN^- was on the acid secretory mechanism. However, since Forte and Davies (2) have reported SCN^- inhibition of oxygen consumption in intact mucosal sheets it seems desirable to determine whether SCN^- can inhibit respiration in isolated, functionally intact mitochondria.

Materials and Methods. Attempts to prepare mitochondria from bullfrog gastric mucosae and bullfrog liver were unsatisfactory, as judged by the P/O ratio and respiratory control ratio of the particles obtained. Therefore, a standard rat liver mitochondria preparation (3) was employed, in which the mitochondria were reasonably intact by these criteria.

The final mitochondrial pellet was suspended in 0.25 *M* sucrose–0.01 *M* phosphate

* Supported by NSF (GB-4074) and Research Corporation grants.