PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 160, 1–5 (1979)

Inconsistency of Cell-Mediated Immunological Assays when Viral Reagents Contain Endotoxin[1] (40376)

ROBERT M. DONAHOE AND ROBERT L. PETERS

Microbiological Associates, Walkersville, Maryland 21793

Endotoxins of gram-negative bacteria frequently contaminate viruses produced in cell culture and consequently are found in various commercially available viral vaccines (1). Because it is difficult or at times impractical to eliminate endotoxin contamination, especially in large scale production efforts, its presence in a variety of biologicals has often been tolerated or unknown. Endotoxins are potent immunologic and physiologic regulators (2, 3), and even subnanogram amounts in commonly available chemicals can induce inflammatory responses (4). In using type-C RNA viral reagents for experimental application of previously developed (5, 6) cell-mediated immunologic assays, it was noticed that undesireable inter-assay variation correlated with the use of various reagent lots which had been produced in cell culture. Thus, the hypothesis was suggested that endotoxin might be present in the virus-containing material and that it might be responsible for alteration of assay results.

Studies reported here were undertaken to determine whether endotoxin was present in the viral reagents used in a lymphocyte transformation assay and a footpad swelling assay and whether its presence could cause assay results to vary.

Materials and methods. Viruses. Rauscher murine leukemia virus (R-MuLV), Simian sarcoma virus (SSV), Gross murine leukemia virus (G-MuLV), AKR-murine leukemia virus (AKR-MuLV) and feline leukemia virus (FeLV) were obtained through the Resources and Logistics Branch of the Virus Cancer Program, National Institutes of Health, Bethesda, MD. Mason-Pfizer monkey virus (MPMV) was obtained as a gift. Each virus had been banded twice in sucrose and then pelleted, and was received suspended in 0.0167 *M* sodium citrate. The final virus concentration was 1000- to 5000-fold that of the original. Protein concentrations were determined for each virus preparation by the method of Lowry *et al.* (7).

Virus reagent production. Vaccines were prepared from the virus preparations by inactivation with formalin (5, 6). UV-irradiated R-MuLV was used as an eliciting antigen in the *in vitro* phase of the lymphocyte transformation assay (LTA) (5).

Mice. BALB/cCr mice were obtained from Microbiological Associates, Walkersville, MD, and BALB/cAnN mice from the National Cancer Institute Animal Farm at the Frederick Cancer Research Center, Frederick, MD.

Cell-mediated immunological assays. The LTA for measurement of sensitivity to MuLV has been described previously (5). Fourteen days after vaccination of mice with Formalin-inactivated virus, spleens are removed from five mice from each experimental group, pooled, and mashed with the blunt end of a sterile syringe in RPMI-1640 medium supplemented with 10% human serum and antibiotics (cell culture reagents from Microbiological Associates, Bethesda, MD). The cells are filtered through gauze, washed and suspended to 5×10^6 leukocytes/ml culture medium. Replicates (0.1 ml) of each suspension are cultured in #3040 Microtest II tissue culture plates (Falcon Plastics, Oxnard, CA) along with 0.05 ml of antigen, mitogen or, as a baseline control, culture medium. One day prior to harvest, each well is pulsed with 0.5-μCi [^{3}H]thymidine (New England Nuclear Corp., Boston, MA; sp. act. 2 Ci/m*M*). Replicate sets of cultures are harvested after 3 or 5 days. Incorporation of radioactive label into DNA is determined. Stimulation of cell division is calculated as a mean stimulation index by the following formula:

[1] This work was supported by Contract No. N01-CP-33248, sponsored by the National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

0037-9727/79/010001-05$01.00/0

$$\text{Mean Stimulation Index} = \frac{\text{Mean cpm of radioactive label incorporated by leukocytes from test or control mice exposed to antigen or mitogen}}{\text{Mean cpm of radioactive label incorporated by leukocytes from test or control mice exposed to culture medium}}$$

The footpad swelling assay (FSA) for detection of delayed-hypersensitivity responses to MuLV has been described previously (6). Seven days after vaccination with formalin-inactivated virus, mice are challenged in the left hind footpad with 15 μl of antigen or control preparation. Right hind footpads are inoculated with diluent as controls. The hind footpads are then measured with calipers 4 and 24 hr after challenge. A percent mean increase in footpad diameter per test or control group (5–6 mice each) is calculated by dividing the mean difference in left and right footpad diameters of each group by the mean diameter of the control footpad.

The standard endotoxin used in these assays was from Difco, Detroit, MI, *Escherichia coli* lipopolysaccharide 0127:B8, Westphal extract.

Endotoxin determinations. The Limulus Amebocyte Lysate (LAL) test kit (Microbiological Associates, Walkersville, MD), with a sensitivity of 0.25-ng endotoxin/ml, was used for measurement of endotoxin. In preliminary studies it was determined that the type-C RNA virus, when present, did not interfere with the measurement of endotoxin with this assay.

Results. Representative vaccine lots were examined for endotoxin. Endotoxin concentrations ranged from 5 to 2500 ng per mg of virus protein (Table I). Other virus lots, including UV-irradiated R-MuLV, that had not been formalin treated for use as vaccines also contained various levels of endotoxin.

A study to determine the effects of various levels of endotoxin in viral reagents on the LTA was conducted. A R-MuLV lot, containing low levels of endotoxin (2.5-ng endotoxin/mg virus protein) was divided and one portion inactivated with formalin while the other was UV-irradiated as previously described (5). Each portion was then supplemented with endotoxin so that final endotoxin concentrations were either 50 or 500 ng/mg virus protein. These preparations were used as formalin-treated virus vaccines or UV-irradiated viral antigens in a LTA. The outline and results of this assay which is representative of data from several similar studies are shown in Table II. Each stimulation index recorded was determined for five replicate cultures. The background counts which are not shown were comparable for each vaccine or vaccine-control group.

TABLE I. ENDOTOXIN IN TYPE-C RNA VIRUS VACCINES.

Virus vaccine	Endotoxin levels in representative type-C RNA virus vaccine preparations
R-MuLV	500,[a] 400, 400, 40, 40, 40, 5, 5, 5
G-MuLV	666, 500, 500, 400, 400, 400, 400, 40, 5
AKR-MuLV	400, 400, 400
FeLV	1000
SSV	<100
MPMV	2500

[a] ng endotoxin per mg virus protein. Endotoxin determined by LAL assay.

Virus-specific stimulation of sensitized leukocytes from vaccinated mice was demonstrable as reported previously (5). The effects of endotoxin on the LTA were manifest in two ways: (a) When endotoxin was present in the formalin-treated virus vaccine in relatively high concentrations (500 ng/mg virus protein), responses in the LTA to virus antigen were depressed, especially when the spleen cells were cultured for 3 days; (b) When relatively high concentrations of endotoxin were present *in vitro* in the UV-irradiated viral antigen, virus-specific responses in the LTA were enhanced, if cultures were derived from adequately sensitized mice, i.e., from mice that received virus vaccines containing 2.5- or 50-ng endotoxin. Virus-associated endotoxin in the 500-ng endotoxin per mg virus-protein vaccine seemed to sensitize mice so that their lymphocytes responded to *in vitro* exposure to endotoxin alone. Otherwise, other concentrations of endotoxin did not induce primary or secondary immune responses. Consistent trends in the lymphoblastic responses were apparent in that 5-day cultures from mice adequately sensitized to virus were stimulated by virus better than 3-

TABLE II. EFFECTS OF ENDOTOXIN ON THE LYMPHOCYTE TRANSFORMATION ASSAY FOR MURINE LEUKEMIA VIRUS ANTIGENS.

Vaccine group	Spleen cells used for LTA were derived from mice vaccinated with:[a]	Duration of spleen cell culture (days)	Mean stimulation indices determined from spleen cells cultured with: 5-μg R-MuLV plus endotoxin 0.0125 ng	0.25 ng	2.5 ng	Endotoxin 0.0125 ng	0.25 ng	2.5 ng	5000 ng
Test A	R-MuLV vaccine containing 2.5-ng endotoxin/mg protein	3	3.86	3.84	6.44	1.54	1.35	1.78	n.t.[b]
		5	5.85	6.21	9.76	1.64	1.50	1.54	2.72
Test B	R-MuLV vaccine containing 50-ng endotoxin/mg protein	3	5.78	5.62	6.69	1.19	1.46	1.89	6.60
		5	6.29	6.40	8.58	1.33	1.25	1.58	2.41
Test C	R-MuLV vaccine containing 500-ng endotoxin/mg protein	3	2.06	2.23	2.70	1.18	1.78	2.02	3.71
		5	2.27	1.92	2.26	1.01	1.24	1.06	1.83
Control A	Endotoxin concentration equivalent to that in vaccine group Test A	3	1.16	1.16	1.60	1.22	1.40	1.75	2.73
		5	0.88	0.70	0.73	0.72	1.53	1.33	2.01
Control B	Endotoxin concentration equivalent to that in vaccine group Test B	3	1.74	1.84	1.87	1.07	1.70	2.27	4.65
		5	1.18	1.31	1.12	0.86	1.01	1.31	2.20
Control C	Endotoxin concentration equivalent to that in vaccine group Test C	3	1.71	2.16	2.11	1.32	1.81	2.17	4.68
		5	1.18	1.00	1.36	1.14	1.36	1.12	1.61
Control D	Complete Freund's adjuvant and vaccine diluent	3	1.21	1.32	1.66	1.19	1.29	1.34	2.84
		5	1.56	1.43	1.53	1.02	1.20	1.22	1.48
Control E	Vaccine diluent	3	1.23	1.60	2.04	1.44	1.78	2.26	3.60
		5	1.18	1.36	1.17	1.45	1.25	1.29	1.66

[a] Vaccines were injected, 0.1 ml subcutaneously, on a schedule of 0, 7 and 14 days. The first vaccination incorporated 200-μg protein-doses of virus vaccine mixed 1:1 with complete Freund's adjuvant (CFA); the second and third doses of vaccine incorporated 100-μg protein-doses of virus vaccine. This vaccine regimen was shown previously (5) to be optimal for induction of LTA-detectable sensitivity in BALB/cCr mice. Control preparations were injected on the same schedule and all, except for Control E, included CFA mixed 1:1 for the first injection. Spleen cells were harvested for the LTA 14 days after administration of the last vaccine.

[b] n.t. = not tested.

day cultures, while the reverse was generally true when control cultures were stimulated by endotoxin alone.

A study to determine the effects of various levels of endotoxin in viral reagents on the FSA was conducted. A 30-μg dose of either R-MuLV vaccine, containing 5-ng endotoxin, or G-MuLV vaccine, containing 500-ng endotoxin per mg virus protein, was injected subcutaneously into 8- to 10-week-old mice. The outline and results of this experiment appear in Table III and are representative of other similar studies.

Virus-associated endotoxin in the formalin-treated virus vaccines altered the outcome of the FSA. A relatively high concentration of endotoxin (500 ng/mg virus-protein) in the vaccine depressed its ability to induce measureable virus-specific immunity. Lower concentrations of endotoxin (5 ng/mg virus protein) did not depress induction of immunity. The participation of virus-associated endotoxin in induction of secondary immune responses after injection of footpads was difficult to delineate. The higher endotoxin dose, whether injected alone or with virus, induced generally prominent footpad swelling (5–10%) that was usually greater 4 hr after injection than 24 hr. The lower endotoxin dose, when injected with virus, induced slight (2–7%) footpad swelling in mice inoculated previously with any of the vaccines or vaccine-controls excluding those inoculated with diluent alone, which showed no response. In these instances footpad swelling was again most prominent at 4 hr. When the lower dose of endotoxin was injected alone into footpads, slight (2–5%), 4-hr responses

TABLE III. FOOTPAD SWELLING RESPONSE OF MICE VACCINATED AND CHALLENGED WITH MuLV VACCINES CONTAINING RELATIVELY HIGH OR LOW AMOUNTS OF ENDOTOXIN.

	Mean percentage increase in footpad thickness in response to challenge antigens[b]							
	15-μg R-MuLV with 0.08-ng endotoxin		15-μg G-MuLV with 7.5-ng endotoxin		Endotoxin			
					0.08 ng		7.5 ng	
Vaccine[a]	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr
30-μg R-MuLV, containing 0.15-ng endotoxin + CFA[c]	2 ± 2	17 ± 7	8 ± 3	10 ± 5	8 ± 5	8 ± 5	5 ± 2	3 ± 2
30-μg G-MuLV, containing 15.0-ng endotoxin + CFA	7 ± 4	0	8 ± 3	3 ± 2	3 ± 0	1 ± 2	5 ± 2	1 ± 2
0.15-ng endotoxin + CFA	4 ± 2	0	6 ± 4	7 ± 4	4 ± 2	8 ± 4	2 ± 0	3 ± 2
15.0-ng endotoxin + CFA	5 ± 2	2 ± 3	5 ± 4	3 ± 0	3 ± 1	1 ± 0	10 ± 6	4 ± 3
CFA (Control)	5 ± 3	6 ± 5	8 ± 4	2 ± 1	5 ± 3	9 ± 4	5 ± 2	1 ± 2
Diluent (Control)	0	2 ± 1	8 ± 1	5 ± 4	2 ± 1	2 ± 2	5 ± 3	3 ± 3

[a] Vaccines were suspended in sodium–citrate diluent and mixed 1:1 with CFA. Groups of 20, 8- to 10-week old BALB/cCr mice were inoculated subcutaneously with 0.1 ml of each vaccine.

[b] Five mice from each vaccine group were tested per footpad challenge antigen.

[c] CFA = complete Freund's adjuvant.

predominated; however, a fairly high response (8%) was seen in mice vaccinated with R-MuLV that contained 5-ng endotoxin per mg virus protein. The 24-hr response to the lower dose of endotoxin also was high (8%) in mice vaccinated either with R-MuLV plus 5-ng endotoxin per mg virus protein, an equivalent dose of endotoxin alone, or CFA alone. The CFA was the only common component in these latter three vaccines. The 24-hr responses of mice to the other vaccines in this instance were negligible (1–2%). These results were further corroborated with findings from other studies (data not shown) in which endotoxin alone was repeatedly shown to induce extensive footpad swelling 4 hr after exposure, even with subnanogram doses.

Discussion. The results indicate that endotoxin can be a frequent contaminant of type-C RNA viruses grown in cell culture. Similar contamination has been observed in other virus vaccines (1) and a variety of biologicals (4, 8). Animal sera used for virus propagation are the likely sources of endotoxin-contaminated virus reagents (9, 10). However, other sources cannot be discounted. Viruses used were of the lipid-enveloped type, which might be expected to attract and integrate lipophilic molecules of endotoxin. Also, they were concentrated 1000- to 5000-fold. These facts could account for the relatively high levels of endotoxin present, from 5 to 2500 ng per mg virus-protein. The use of the LAL assay facilitated endotoxin determinations. Untreated, formalin-treated and UV-irradiated virus preparations were shown to contain endotoxin with this assay. Whether these treatments qualitatively altered endotoxin is not known. The need for qualitative analysis of the endotoxin in these reagents is somewhat obviated by the fact that as contaminants these endotoxins would be difficult to type, especially when multiple contaminants could be involved. Because of these considerations, endotoxin values determined for the virus reagents with the LAL assay should be regarded as approximations.

Endotoxin is a well-known immunoregulatory agent. However, concentrations below 5–10 ng/ml have not been regarded as problematic for most immunologic assays. There has been one recent report drawing attention to this matter (11). The present results show that subnanogram amounts of endotoxin can induce variation of responses in a LTA and a FSA. Several reasons can be sited to support the conclusion that endotoxin in the virus reagents was the responsible immunoregulator. Controls implicated the participation of endotoxin. Dosimetry of the responses indicated that endotoxin was responsible. No other probable virus-reagent contaminant like serum proteins or cell components could be expected to have the observed effects.

Complete elucidation of the immunoregulatory effects of endotoxin was not practical

in this study. The data qualify previous reports on the development of the LTA (5) and the FSA (6) with type-C RNA virus reagents. In the LTA, endotoxin did not interfere with demonstration of virus specificity under optimal conditions. The same was true for the FSA. There were however noticeable enhancing and suppressing effects exhibited by endotoxin in both assays under the proper conditions. Adjuvant and immunosuppressive properties of endotoxin have been reported before (12–14). Immunoregulation by endotoxin in the FSA was more variable than in the LTA. The extent of prior virus specific immunity and possibly immune crossreactivity between endotoxin and CFA may have contributed to this observation. Based on these findings it seems logical to propose that proper controlled adjustment of endotoxin levels with test antigens in LTAs could improve the sensitivity of this technique, and that the FSA might serve as an alternate ultrasensitive *in vivo* assay for endotoxin itself, perhaps analogous to the assay of Bito (4) for measurement of endotoxin-induced inflammation of rabbit eyes.

The presence of endotoxin in type-C RNA viruses is of particular theoretical concern because of the biology of the virus involved. Recent studies (15–18) have shown that endogenous type-C RNA viruses can be induced by endotoxin from lymphocytes *in vitro*. For virus induction, from 10 to 100 μg of endotoxin per 10^6 to 10^7 lymphocytes is required. The virus vaccines used in this and other (5, 6) studies were administered in CFA. Because CFA induces sequestration of antigens and infiltration of mononuclear cells (19), endotoxin-lymphocyte concentration ratios similar to those reportedly required for type-C RNA virus induction *in vitro* could also have been achieved in the aforementioned *in vivo* studies. The immunological consequences of this potential virus induction could be significant. Specificities of responses could be erroneously determined and other suggested biological effects of virus injection misinterpreted. Investigators in the area should consider these factors and monitor reagents to avoid the problems involved.

Summary. Endotoxin in concentrations of 5–2500 ng per mg of virus protein, was found in type-C RNA virus reagents. Cell-mediated immune responses induced with these endotoxin-contaminated reagents were evaluated by the lymphocyte transformation assay and the footpad swelling assay. The presence of even subnanogram concentrations of endotoxin altered assay results. Both immunoenhancement and immunosuppression was observed.

The authors thank Mr. R. Van Vleck, Mr. A. Beasley, Mrs. M. Banks, and Mrs. B. Staley for technical assistance; and Drs. J. Djeu, K.-Y. Huang, P. Kind, B. Sass, and J. Parker for their helpful comments.

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Received July 28, 1978. P.S.E.B.M. 1979, Vol. 160.