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Comparison of Rates of Virus Isolation from Leukocyte Populations Separated from Blood by Conventional and Ficoll-Paque/Macrodex Methods

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One hundred fifty-two blood specimens, largely from immunocompromised patients, were collected in heparinized Vacutainer tubes and divided into paired aliquots of equal volume. Buffy-coat preparations, containing mixed leukocyte and separate mononuclear and polymorphonuclear leukocyte populations were obtained by treatment of blood with conventional and Ficoll-Paque/Macrodex (F-P/M) methods. The development of cytopathic effect in monolayers of WI-38 fibroblasts inoculated with cell suspensions derived from the two methods was used to assess virus infectivity. Twice as many virus isolations were obtained using F-P/M. Of those viruses isolated by both conventional and F-P/M, the development of cytopathic effect was more extensive using the latter method. Moreover, a greater variety of viruses was isolated using F-P/M method, as compared to the conventional method. The F-P/M method is no more time consuming than conventional procedures, is readily adaptable for use in the diagnostic virology laboratory, requires only minimal additional cost, and is a particularly suitable and effective means of monitoring viremia.

The detection, surveillance, and control of infection in the immunocompromised host is a difficult and continuing problem. In these patients, infection often eventuates in severe and disseminated disease or death and is virtually unavoidable. Although bacteria and fungi predominate as the major etiological agents in the immunocompromised host, a significant proportion of morbidity and mortality is attributed to infection with viruses. For example, cytomegalovirus (CMV) is emerging as the major etiological agent in renal transplant, bone marrow transplant, leukemia, and aplastic anemia patients, and a variety of other conditions associated with increased susceptibility to infection, from both endogenous and exogenous sources (5, 6, 8, 12, 15, 18, 19).

Continuing improvements in technology are enhancing the laboratory's ability to effectively monitor the efficacy of new therapeutic regimens and modalities (4, 14) and to delineate more accurately the essential elements involved in the maintenance and continuance of the chain of transmission of infectious agents. In this paper, data are presented on a prospective comparison of virus isolation rates from buffy-coat preparations, largely from immunocompromised hosts, using both conventional and Ficoll-Paque/Macrodex (F-P/M) methods.

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MATERIALS AND METHODS

Patients. The majority of patients in this study had either aplastic anemia or acute leukemia before entering the UCLA Bone Marrow Transplant Program. Urine, throat, and heparinized blood specimens for virus culture and serum specimens for serological studies were collected at regular intervals.

Specimens. One hundred fifty-two heparinized blood specimens were collected in 7-ml Vacutainer tubes and divided into paired aliquots of equal volume. Paired aliquots were processed simultaneously by both conventional and F-P/M methods.

Conventional leukocyte harvesting. Heparinized blood was allowed to sediment at room temperature for 1 to 2 h. The plasma layer containing mixed leukocyte populations, consisting of mononuclear and polymorphonuclear leukocytes, was aspirated and centrifuged at 160 × *g* for 5 to 10 min. Cell pellets were suspended in 0.5 to 1.0 ml of plasma.

F-P/M leukocyte harvesting. Heparinized blood was diluted with an equal volume of 0.9% NaCl and layered onto Ficoll-Paque solution (Pharmacia, Piscataway, N.J.) in a ratio of 3:1 in 17- by 100-mm sterile, plastic tubes with snap caps (Falcon Plastics, Oxnard, Calif.). Specimens were centrifuged at 400 to 440 × *g* for 30 min at room temperature in a bench-top model IEC clinical centrifuge (Damon/IEC Division, Needham Heights, Mass.). The mononuclear fraction which

lay above the Ficoll-Paque gradient was removed, washed twice in Eagle minimum essential medium containing 5% fetal calf serum, and suspended in 2 ml of Eagle minimal essential medium (3, 10).

Erythrocytes and polymorphonuclear leukocytes (PMNs) which sedimented below the Ficoll-Paque gradient were mixed with 6% (wt/vol) Dextran 70 in normal saline (Macrodex, Pharmacia, Piscataway, N.J.) in a 2:1 ratio and allowed to settle at room temperature for 60 min in an inverted sterile syringe (3, 7). The PMN-enriched supernatant was separated from the sedimented erythrocytes, washed twice in Eagle minimal essential medium containing 5% fetal calf serum, and inoculated into cell culture.

Specimen inoculation and virus isolation. Human embryonic lung fibroblasts, WI-38, were purchased in 16- by 125-mm glass tubes from Flow Laboratories (Rockville, Md.). Maintenance medium consisted of Eagle minimal essential medium supplemented with 5% fetal calf serum, 7.5% sodium bicarbonate, penicillin, streptomycin, kanamycin, and mycostatin. Media in all cell cultures were changed twice weekly.

Mixed leukocyte populations obtained from conventional harvesting were inoculated in duplicate onto WI-38 monolayers, 0.2 to 0.5 ml per tube (cell concentrations ranged from 10^4 to 10^6 cells per ml). After adsorption for 1 to 2 h at room temperature, inocula were removed and cultures were fed with fresh medium.

Mononuclear and PMN leukocytes, isolated by the F-P/M method, were inoculated, in duplicate, onto WI-38 monolayers, 1 ml per tube (cell concentrations averaged 10^6 cells per ml). After overnight adsorption at 37°C, inoculated cells were removed and monolayers were washed and fed with fresh medium.

Cell cultures were checked for the development of typical cytopathic effect twice weekly for 6 weeks. Isolates were identified by type of cytopathic effect produced, incubation period, hematoxylin and eosin staining of infected cells, and/or neutralization tests with specific antisera.

Enumeration and determination of viability. Leukocyte viability, as estimated by trypan blue exclusion, averaged 98% using both conventional and F-P/M methods. Harvested leukocytes, diluted in Turk solution and counted on a Spencer Bright-Line hemacytometer, yielded counts ranging from 10^3 to 10^6 cells per ml.

RESULTS

Of the 152 blood specimens processed, virus strains were isolated from 17 over a 5-month period (Table 1). Eight of these isolates were detected by conventional techniques, whereas 16 were recovered using the F-P/M method. Twelve of 15 isolates were recovered from the PMN fraction, and 8 of 16 were recovered from the mononuclear fraction. Six of 16 were obtained from the PMN fraction only, and in three instances virus was recovered only from the mononuclear fraction. In only one instance was a virus isolated by the conventional method alone, whereas there were nine instances in which recovery of virus was obtained only by the F-P/M method.

TABLE 1. *Numbers and types of viruses recovered using conventional and F-P/M leukocyte harvesting methods*

Method	No. of viruses isolated[a]	Types of viruses isolated[b]
Conventional (C) (mixed leukocytes)	8 (1)	CMV (8)
Ficoll-Paque (F-P) (mononuclears)	8 (3)	CMV (6) Adenovirus (1) Varicella (1)
Macrodex (M) (PMNs)	12 (6)	CMV (12)
Total		
F-P/M	16 (9)	CMV (14)
C+F-P/M	17	Adenovirus (1) Varicella (1)

[a] Number in parentheses indicates number of virus isolates recovered only by this treatment.

[b] Number in parentheses indicates number of strains of each kind of virus isolated.

Utilization of the F-P/M method resulted in a larger yield of viruses, both qualitatively and quantitatively, as compared to conventional techniques. CMV was the most common virus isolated; 14 of the 16 viruses isolated by F-P/M were identified as CMV. CMV was obtained from both the mononuclear and PMN fractions. Adenovirus type 3 and Varicella-Zoster virus were each isolated once, and both were recovered from the mononuclear fraction only, using the F-P/M method. The Varicella-Zoster virus isolate was obtained simultaneously with a CMV from the same specimen. CMV was the only virus isolated using conventional procedures.

The average number of days required to detect cytopathic effect was not significantly different overall (Table 2). However, it should be noted that, in nine instances, recovery of virus was obtained only from leukocytes harvested by the F-P/M method.

The average yield of infectious foci or plaques utilizing the F-P/M method was greater than that detected by the conventional method (Table 3). The average number of plaques obtained by the conventional method did not differ significantly from that derived from the mononuclear fraction. However, the yield from the PMN fraction was more than six times that obtained with the conventional method and was nearly five times that of the mononuclear fraction.

The recovery rate of viruses using the conventional method was 5.2%, whereas that for F-P/M was 10.5%; this represents a 100% increase

TABLE 2. *Length of time for development of cytopathic effect using conventional and F-P/M leukocyte harvesting methods*

Patients	Time needed for CPE development[a]			Virus isolated[b]
	Conventional (mixed leukocytes)	Ficoll-Paque (mononuclears)	Macrodex (PMN)	
1	10*	27	ND	CMV
2	N	N	14	CMV
3	N	N	33	CMV
4	25	28	28	CMV
5	45	N	N	CMV
6	21	21	21	CMV
7	N	N	28	CMV
8	N	N	22	CMV
9	20	14	17	CMV
10	20	NA	10	CMV
11	12	18	12	CMV
12	N	24	N	CMV
13	N	13	N	Adenovirus type 3
14	N	N	31	CMV
15	N	N	19	CMV
16, 17	20 (CMV)	12 (V-Z)	21 (CMV)	CMV, VZV
Mean time	21.6	19.6	21.3	

[a] Days elapsed before initial detection of typical viral cytopathic effect (CPE): ND, not done; NA, not applicable, cell culture lost; N, cytopathic effect not noted.
[b] VZV, Varicella-Zoster virus.

over the conventional method. In a retrospective analysis of virus isolations over an 8-year period using conventional procedures, it was found that an overall recovery rate of 2.8% was obtained from buffy-coat preparations, which is less than one-third that obtained by F-P/M.

As determined by the trypan blue dye exclusion test, the viability of the mixed-leukocyte population harvested by the conventional method and that of separate mononuclear and PMN fractions varied from 98 to 100%. Repetitive enumeration of leukocyte yields from conventional and F-P/M methods revealed that the latter method generally resulted in total cell harvests 2 to 10 times that of the former (occasionally 50 times greater).

The F-P/M method was generally no more time consuming than conventional methods, was readily adaptable for use in the diagnostic virology laboratory, and required only minimal additional cost.

DISCUSSION

Recovery rates of CMV in the laboratory vary with the specimen source, patient study group, and the isolation techniques employed. Frequently sampled sources include urine, buffy coat, and respiratory specimens such as throat, sputum, or saliva. Of these sources, urines yield the greatest number of isolates, ranging from

TABLE 3. *Virus isolation and plaque yields from blood processed by conventional and F-P/M leukocyte harvesting methods*

Patient	CMV plaque yield (no.)[a]		
	Conventional (mixed leukocytes)	Ficoll-Paque (mononuclears)	Macrodex (PMN)
1	17	50	ND
2	0	0	40
3	0	0	20
4	Several	3	50
5	11	0	0
6	6	13	120
7	0	0	2
8	0	0	25
9	1	1	1
10	18	NA	8
11	13	3	50
12	0	10	0
13	0	Adenovirus type 3	0
14	0	0	2
15	0	0	2
16, 17	1	V-Z	20
Mean no. of infectious foci	4.5	6.2	28.3

[a] Number of typical CMV plaques, except where other viruses are denoted. VZV, Varicella-Zoster virus; ND, not done; NA, not applicable, cell culture lost.

25% in patients with acute lymphocytic leukemia (5, 12), 20 to 78% in patients with rheumatologic disorders (6), and 47 to 90% renal transplant patients (8, 17). Viremia is not as readily demonstrated, and reported isolation rates range from 0 to 9% in acute leukemia (1, 5), 0 to 46% in renal transplant patients (12), 80% of congenitally infected infants (11), and 100% in persons with acute mononucleosis (16). Differences in reported virus isolation rates from buffy coat specimens reflect not only the nature of the underlying disease in defined patient populations but harvesting procedures as well. Whole blood sedimentation at 37°C (5) or at room temperature (11), followed by aspiration of the leukocyte layer (6) or centrifugation with Ficoll-Hypaque/Dextran (8, 9, 16), and variations of these methods are typical examples of some of the procedures which have been utilized for isolation of leukocytes from peripheral blood.

Methods which can increase virus recovery rates from blood specimens are desirable for diagnosis, prognosis, and monitoring infection, particularly in the immunosuppressed host. It is also useful in determining whether or not there is a requirement for bacterial and fungal antimicrobial therapy which may be potentially toxic and unnecessary. The present study indicates that leukocyte harvesting methods can directly affect virus isolation rates. The F-P/M method yielded twice the virus isolation rate when compared to conventional procedures. In addition to higher virus yields, a greater variety of viruses was detected with the F-P/M technique. Furthermore, application of the F-P/M method resulted in a significantly greater number of infectious foci or plaques, approximately six times that obtained with the conventional procedure. This observation correlates with the higher number of leukocytes harvested with F-P/M, ranging from 2 to 10 times, and occasionally up to 50 times the leukocytes obtained by the conventional procedure. Approximately five times as many infectious foci were observed in cell cultures inoculated with the PMN fraction as in those inoculated with the mononuclear fraction. Recovery of CMV, principally from PMNs, is in agreement with observations made by other investigators in which the virus was isolated more frequently from PMNs than from monocytes and not at all from lymphocytes (16). Other studies have shown that PMNs also yielded higher virus titers than did monocytes or lymphocytes when both fractions contained virus (8, 9). Isolation of virus from PMNs and occasionally monocytes is probably a reflection of the phagocytic function of these cells. It is not clear whether CMV replicates within PMNs or their precursors or whether the virus is simply phagocytized during infection.

The fact that the F-P/M procedure for isolating leukocytes is no more time consuming than the conventional method, requires only minimal additional cost, and is readily adaptable to the diagnostic virology laboratory makes it a particularly suitable and effective means of monitoring viremia.

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